MOLECULAR BASIS OF LIFE-HISTORY EVOLUTION: A TALE OF TWO INSECTS

BY

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DISSERTATION

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ABSTRACT

The field of life-history evolution is aimed at understanding the diversity of fertility and longevity patterns observed in nature. These patterns are influenced by the interplay of traits that directly affect the fitness of individuals, including age at first reproduction, growth rate, age-specific fecundity, and age-specific survival. Variation in life-history strategies occurs because of phylogenetic constraints on organisms, influence of extrinsic factors on mortality (i.e. predation), and tradeoffs in energy allocation between competing physiological processes. Differences in life-history strategies have been well documented at the phenotypic level but their causal genetic mechanisms remain largely unknown.

At the genetic level, tradeoffs between lifespan and reproduction have been hypothesized to arise because the force of natural selection decreases with advancing age and favors pleiotropic alleles that have beneficial effects on reproduction early in life even at the cost of survival later in life. Experimental evolution studies in *Drosophila* have highlighted the existence of tradeoffs between lifespan and reproduction that seem to be consistent with the concept of antagonistic pleiotropy. Selection for increased age at first reproduction, as well as selection for lifespan led to increases in lifespan and reduced early life fecundity in these flies.

The underlying physiological cause of tradeoffs has been difficult to study because of the diversity of processes that influence life-history traits. Given that increases in lifespan have widespread influence on reproductive output, molecular geneticists interested in aging have documented survival costs of reproduction in long-lived individuals. Mutations in signaling pathways that couple environmental signals to key physiological processes affect growth, reproduction and lifespan. These studies have provided molecular mechanisms that are excellent

candidates for regulating life history traits. However, whether natural variation in any of these genes is important in life history evolution remains an open question.

My dissertation research focused on understanding differences in life histories in one eusocial and one non-social insect. Eusocial insects are good candidates to study mechanisms of tradeoffs between fecundity and survival. Eusocial insect queens enjoy a long lifespan that does not come at the cost of reduced fecundity, whereas workers are usually short lived and non-reproductive. Both queens and workers can potentially develop from larvae with identical genotypes but yet show strikingly different phenotypes as adults. My work on honey bee aging established the importance of intrinsic physiological factors in regulating differences in lifespan between queen and worker bees, and provided a potential mechanism for such differences.

For the second part of my dissertation, I generated fruit fly strains with divergent life-histories to study the molecular underpinnings of life-history evolution. These studies were designed to investigate how phenotypic tradeoffs are regulated at the molecular level. I used a candidate gene approach to evaluate the role of insulin signaling in differential survival and reproduction. Results from this study do not support the involvement of genes in this pathway in life history divergence. A genome-wide screen was also employed to evaluate if other genes were involved in regulating the tradeoff between reproduction and lifespan in my fly strains. Genes involved in nutrient reservoir activity, stress response, and detoxification were differentially expressed between strains. This suggests that life history divergence in my fly lines was possible through differential energy allocation to competing processes (i.e. somatic maintenance vs. reproduction).

Understanding variation among organisms in patterns of longevity and reproduction is a key goal of evolutionary biology. Research on the molecular mechanisms that regulate the

evolution of life history traits will allow us to link the genetic architecture of these traits to the ecological factors that shape them and this will ultimately help us understand how organisms adapt to their environment. The study of the mechanistic basis of tradeoffs between lifespan and reproduction is also fundamental given the relationship between aging and other life-history traits.

Dissertation Objectives

Objective 1: Evaluate the influence of intrinsic mortality factors in determining lifespan of worker honey bees.

Worker honey bees of different age classes (10, 30, and 50 days of age) were exposed to three physiological stress treatments: starvation, heat, and oxidative stress. I compared survival curves of bees under each stressful treatment to investigate if younger bees tolerated physiological stress better than older bees. Differences in lifespan between queen and worker bees had previously been attributed to risks associated with foraging behavior in workers (i.e. wear and tear, accident, hazardous weather conditions). I found that worker bees prevented from foraging, show an age-related physiological deterioration in performance. This suggests that differences in lifespan between queens and workers are influenced by intrinsic physiological mechanisms.

Objective 2: Examine the involvement of the insulin-signaling pathway in the regulation of tradeoffs between lifespan and reproduction.

I used experimental evolution to generate fly lines with divergent life histories: high early life fecundity/short lifespan vs. low early life fecundity/long lifespan. Using quantitative real time PCR, I measured differences in mRNA expression between my fly lines at three different

age classes (14, 44, and 60 days of age) for five genes in the insulin-signaling pathway. Candidate genes were chosen based on previous studies where mutations in these genes were shown to alter lifespan and fecundity. I did not find significant differences in expression, suggesting that this pathway is not involved in the evolution of divergent life histories in these flies.

Objective 3: Use a genome-wide screen to identify previously characterized and novel genes involved in the evolution of divergent life-histories in fruit flies.

Previous results did not suggest a role for the insulin-signaling pathway in the divergence of life histories in my fly lines. Therefore, I used microarray analysis to find candidate genes associated with the regulation of tradeoffs between lifespan and reproduction. I found a total of 468 genes show differential expression between my fly lines. These genes are involved in nutrient reservoir activities, response to stimuli (i.e. stress, detoxification, immunity), and in the regulation of transcription and translation. Functional tests need to be implemented in future studies to directly test the association of these genes with differences in lifespan and reproduction in female flies.

To My Family

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TABLE OF CONTENTS

CHAPTER 1

SENESCENCE IN THE WORKER HONEY BEE *Apis mellifera*

* This chapter includes previously published material: (Remolina et al. 2007). Permission has been granted by El Sevier for inclusion in the publication of this dissertation.

1.1. Introduction

Senescence is defined as an age-related decline in physiological function, performance, survival, or reproduction (Finch, 1990). Senescence (often referred to simply as "aging") is a nearly universal feature of multicellular organisms, and appears to occur even in unicellular yeast and bacteria (Lithgow and Kirkwood, 1996; Kirkwood and Austad, 2000). Understanding the biological processes that lead to senescence, and why different organisms senesce at dramatically different rates, is a long-standing problem in both molecular and evolutionary biology (Lithgow and Kirkwood, 1996; Finch, 1990; Rose, 1991; Partridge, 1993; Kirkwood and Austad, 2000).

In some eusocial insects (ants, bees, wasps, and termites), queens and workers of the same species sometimes show a 100-fold difference in lifespan, with reproductive queens having longer lifespans than the non-reproductive workers (Winston, 1987; Keller and Genoud, 1997; Page and Peng, 2001). Strikingly, the long life of social insect queens does not come at the cost of low reproduction: queens of many social insects lay hundreds or thousands of eggs per day throughout their adult life. Their ability to sustain both high reproductive effort and long life makes social insects particularly promising model systems for studies of aging (Parker et al., 2004; Seehuus et al., 2006; Corona et al., 2005; Corona et al., 2007).

In the honey bee, *Apis mellifera,* queens have an average lifespan of 1–2 years and workers have an average lifespan of 15–38 days in the summer and 140 days in the winter (Winston, 1987). Queens and workers are not genetically distinct, so biological differences

between castes are due to gene expression differences that depend on social and dietary cues that individuals experience during development (Corona et al., 2005). Therefore, studying the comparative physiology and molecular biology of queens and workers is an attractive paradigm for investigating proximate mechanisms of lifespan differences (Parker et al., 2004; Corona et al., 2005). However, there is a potential serious flaw in this paradigm: it is currently not known whether caste-specific lifespan differences result from inherent physiological differences in the rate of senescence or, alternately, from caste-related differences in exposure to risk.

In nature, queen bees leave the protected environment of the hive only to take mating flights at 1–2 weeks of age, and possibly once more later in their life, during colony fission. In contrast, workers spend the first 2–3 weeks of adult life mostly in the hive performing tasks such as brood care ("nursing") before shifting to foraging outside the hive for nectar and pollen, making over 10 trips a day, sometimes at distances of up to 2 km (Winston, 1987). Foragers thus experience risks from predation, thermal stress, and physical exhaustion; risks that queens (and pre-foragers, such as nurse bees) do not experience to the same extent. Thus, a plausible hypothesis for the difference in queen and worker lifespan is that workers, once they become foragers, experience high extrinsic mortality, and therefore have a much shorter mean lifespan than queens.

Only a few studies have addressed the question of whether worker bee lifespan is determined by senescence or exposure to extrinsic risk. Neukirch (1982) compared lifespans of foragers with different amounts of flight experience and found that lifespan was inversely related to daily flight experience. She argued that foragers have fixed energy reserves, and, once the reserve is depleted, foragers cannot fly and fail to return to the hive. This idea does not require physiological senescence. In contrast, later studies found patterns consistent with senescence.

Schmid-Hempel and Wolf (1988) found that workers had fixed lifespans regardless of energy expenditure, and Visscher and Dukas (1997) found that behavioral and foraging performance declined after 10 days of foraging (see also Tofilski, 2000). A limitation of all these studies is that age-specific survival data were collected on foragers, and so were possibly confounded by the cumulative effects of energy expenditure and foraging activity. Because of the lifestyle of the forager, age-related increases in mortality rates could be due to accumulation of injuries or exhaustion of energy reserves, which are not necessarily due to intrinsic physiological deterioration.

We exploited the honey bee's strong plasticity for division of labor (Robinson, 1992) to remove the confounding effects of energy expenditure and risks associated with foraging. Worker bees respond to changing social conditions by accelerating, delaying, or reversing their typical pattern of behavioral maturation. For example, if there is a shortage of foragers or large numbers of young larvae in the hive, some bees delay their transition to foraging and become "overage" nurses (Robinson et al., 1989). We studied age-specific stress resistance in overage nurses that did not experience the extrinsic risk factors associated with foraging. We predicted that if there is worker senescence, then older bees should have lower survival under each stress treatment than younger bees.

1.2. Materials and methods

1.2.1. Experimental colonies

We set up five single-cohort colonies (Robinson et al., 1989), each initially composed of ca. 10,000 1-day-old bees. We obtained 1-day-old worker bees by removing frames of pupae from typical field colonies (headed by naturally mated queens) and placing them in an incubator

(34 °C and 80% relative humidity). The bees were marked with a paint dot on the dorsal thorax, color coded according to day of emergence and source colony. This process was continued over a 5-day period for each colony to obtain the 10,000 bees. Each single-cohort colony was then given a (naturally mated) queen, 4 frames of honey and pollen, and 2 frames for the queen to lay eggs in. We encouraged the development of overage nurses by removing frames of brood prior to the emergence of new adult bees, and replacing them with frames of younger brood.

1.2.2. Collections of bees

At each collection date, we collected 300 bees from each age class (10, 30, and 50 days old) that was available at that date. We collected bees that were displaying typical nursing behavior (head in cell containing a larva; see Huang and Robinson (1996)). Collections were made when foragers were out of the hive during times of active foraging to minimize the chances of misidentification. The five single-cohort colonies were set up in a time-staggered design, so that bees of different age classes were available on the same day (Fig. 1). We were thus able to evaluate the effects of age on stress resistance, and decouple these effects from the effects of source colony and date of collection (seasonality). Bees were held individually in cages within a plexiglass tray, provided with 50% sucrose solution ad lib, and kept at constant temperature (34 °C) for 24 h, prior to the start of the stress tests. After 24 h, the surviving bees were randomly assigned to the three treatment groups. Total sample sizes for each age class and treatment group are given in Table 1.

1.2.3. Stress tests

To detect senescence, we measured the effects of oxidative stress, heat stress, and starvation on bees from each age class. Resistance to these stressors typically declines in senescing insects, causing increased mortality (Luckinbill et al., 1984; Rose, 1984; Nghiem et

al., 2000). After the collections were made, during the next 24 h, bees were housed in an incubator at 34 °C and were provided a 50% sucrose solution so that they could feed freely. After that, bees of the same age class that were still alive were randomly assigned to three different trays and one of the three trays was assigned to each one of the treatments (starvation, heat stress, or hydrogen peroxide). Treatment details are as follows. *Hydrogen peroxide*: Bees were given a 50% sucrose solution that contained 20% hydrogen peroxide. This dose was based on results from *Drosophila melanogaster* that showed that a dose of 5% hydrogen peroxide produced high mortality (Sun and Tower, 1999), adjusting for differences in body mass between honey bees and fruit flies. *Heat stress*: We exposed bees to 42 °C in an incubator; colonies typically maintain their hives at approximately 34 °C by behavioral thermoregulation, and it has been reported (Mardan and Kevan, 2002) that bees kept at 42 °C showed decreased longevity. Bees were kept at 42 °C until they died. *Starvation*: Bees were maintained in an incubator without any food at 34 °C, and were provided with water to prevent desiccation. In all treatments, bees were housed in individual cages within a plexiglass tray. With the exception of the starvation treatment, food was provided in the tray, and bees were allowed to feed freely. Food in the trays was replaced every 6 h, and water was replenished for the bees in the starvation treatment. Food replacement was of special importance for the hydrogen peroxide treatment, since hydrogen peroxide degrades in water. Bees in all incubators were maintained in a 24-hour dark cycle; the hive is naturally dark, except for whatever light penetrates from the hive entrance.

1.2.4. Censusing mortality

Bees were censused at 0:00, 6:00, 12:00, and 18:00 h until all were dead. Information on age and source colony was obtained from the thorax markings. Six bees escaped during the experiment (3 in the heat stress and 3 in the starvation treatment) the escape time of these bees

was treated as a right-censored observation in the data analysis.

1.2.5. Lipid analysis

Because the most striking differences in age-specific stress resistance were observed in the starvation test (see Results), we explored whether the results could be explained by differences in lipid reserves. We measured the abdominal lipid levels of young and overage nurses, using foragers as a comparison group, since foragers have the lowest lipid levels among worker bees (Toth and Robinson, 2005). We used young nurses less than 7 days of age (n=23), 50-day-old nurses (n=23) and 50-day-old foragers (n=22). Each abdomen was dissected and the digestive tract and sting apparatus removed; abdomens were then freeze-dried, homogenized in a 2:1 chloroform:methanol solution, and dried down to a constant volume of 2 ml. The lipid assay was performed using 100 µl of each sample, following the procedures in Toth et al. (2005). We measured the absorbance of each sample using a SpectraMax 190 spectrophotometer (Molecular Devices, CA), with readings at 525 nm. Absorbance readings were converted to milligrams of lipid using a cholesterol standard. The lipid assay was performed twice on each sample.

1.2.6. Data analysis

We calculated Kaplan–Meier (product-limit) survival estimates for the 10-, 30-, and 50-day-old workers for each stress treatment. We tested for differences in survival among age classes within a treatment using the log-rank and Wilcoxon tests produced by SAS Proc Lifetest (SAS System v.9.1). Wilcoxon tests are more sensitive to differences in survival occurring earlier in the trials, while log-rank tests are more sensitive to differences that occur later (Allison, 1995). Results of both tests were consistent in every case, so we report only the log-rank test results. We also tested for significant differences between age classes using Cox proportional hazards models as implemented in SAS Proc Phreg. This test allowed direct comparison of the

hazard rate (risk of death per unit time) for each age class within a treatment group, and formal statistical tests for pairwise differences in hazard rates between age classes (Allison, 1995). In this analysis, a hazard ratio >1 indicates a higher hazard for the older bees, and a value <1 indicates a lower hazard for the older bees. We repeated the pairwise contrast analysis after removing data for colonies 4 and 5; because these colonies are represented by two (or one) age classes, there is a possibility of confounding age and colony effects. For the analysis of lipid data, we treated the replicate measures for each sample as repeated measures in a general linear model (repeated measures ANOVA) using SAS Proc Mixed (Littell et al., 2002).

1.3. Results

Mean survival times for 10-day-old bees were longer than for older bees in all three stress tests (Table 1). Survival curves show that 10-day-old bees had higher survival at each time point than did 50-day-old bees (Fig. 2). Log-rank tests of survival times indicated that age classes differed significantly for the starvation (χ^2=202.6, p<0.0001) and heat stress treatments (χ^2=20.9, p<0.0001), but not for the hydrogen peroxide treatment (χ^2=2.6, p=0.27). However, the semi-parametric tests of the proportional hazards model indicated that differences in hazard rates between age classes were significant for all three treatments: starvation (χ^2=102.2, p<0.0001), heat stress (χ^2=50.82, p<0.0001), and hydrogen peroxide (χ^2=7.8, p=0.0205).

Similarly, pairwise contrasts of the hazard rates within treatments indicated that 10-day-old bees had significantly lower mortality per unit time than did 50-day olds in each treatment (Table 2). All hazard ratio estimates were >1, indicating higher mortality rates for older bees in each comparison; comparisons were significant in 6 out of 9 pairwise tests, and marginally non-significant at P=0.05 in one additional comparison (Table 2). Limiting the analysis to colonies

1–3 produced qualitatively identical results. In this analysis, 10-day-old bees had significantly lower mortality than 50-day-old bees in all three stress treatments (Appendix 1).

Hazard ratios for each pairwise comparison between age classes (ratio of older to younger bees); degrees of freedom=1 in every case. Results in bold indicate significant differences. There were no significant differences in stored lipid in young and old nurses ($F_{[1,64]=}$2.6, P=0.12, Fig. 3). Both young and old nurses had significantly higher lipid content than foragers (young nurses vs. foragers, $F_{[1,64]}$=52.3, P<0.0001; old nurses vs. foragers, $F_{[1,64]}$=31.9, P<0.0001). These results indicate that results of the starvation test are not attributable to differences in stored lipids between young and old nurse bees; overage nurses have lipid levels characteristic of nurses, and not of foragers. These results are consistent with findings from Toth et al., 2005.

1.4. Discussion

Our results provide the first clear demonstration of worker honey bee senescence. In our experiments, this physiological decline began between 10 and 30 days of age and continued through 50 days of age. These results indicate that honey bee workers experience an intrinsic physiological decline at an age that is consistent with their observed maximal lifespan in the summer and their longevity does not depend solely on extrinsic mortality factors.

Our results are unlikely to be due to differences in physical activity because we used overage nurses rather than foragers. It is unlikely that our results, especially for the starvation treatment, can be attributed to older nurses having lower nutritional reserves than younger nurses. Our lipid analysis showed no difference between lipid stores in young and overage nurses, but other nutritional indicators such as glycogen content were not measured. We conclude that the marked

decline in stress resistance in 30- and 50-day-old bees strongly suggests physiological senescence.

Results from the heat stress assay indicated that 30- and 50-day-old bees were more likely to die than 10-day-old bees. Although the differences were highly significant, they were less extreme than in the starvation assay. Perhaps, this is because the treatment was relatively less extreme. Honey bees can tolerate temperatures up to 45 °C for at least 2 h, and humidity is an important factor in their ability to tolerate high temperatures (Free and Spencer-Booth, 1962). Perhaps our treatment was not as stressful as it could have been, because bees were provided with an unlimited source of sugar syrup and full water containers were kept in the incubator at all times.

Differences between age classes in the hydrogen peroxide treatment were relatively small (though statistically significant) compared with the starvation and heat treatments. It seems unlikely that bees in the hydrogen peroxide treatment were not feeding, since the median lifespan of bees of all age classes surpassed that of bees in the starvation treatment. It is possible that the concentration of hydrogen peroxide we used was too weak to induce much oxidative stress or stress-related mortality in our bees. This speculation is supported by the observation that paraquat (another free radical-inducing agent) caused greater mortality in a comparable experiment (Corona et al., 2007). In that experiment, the median lifespan for worker bees 30 days of age was 33 h, compared with 66 h in our experiment. This observation is further supported by another experiment comparing paraquat-induced oxidative stress resistance in worker bees where complete mortality was reached within 60 h of paraquat injection (Seehuus et al., 2006).

Hydrogen peroxide is an oxidizing agent that slowly decomposes into water and oxygen

at room temperature. The decomposition of hydrogen peroxide can be accelerated in the presence of light and at high temperatures, increasing by a factor of 2.2 for every 10 °C rise in temperature. Such decomposition is also catalyzed by dissolved ions of metals, and suspended oxides and hydroxides (Goor et al., 1992). Even though we replaced the hydrogen peroxide and sugar solution in the trays every 6 h, there is a possibility that the decomposition of hydrogen peroxide into water and oxygen may have caused failure to induce mortality in our bees. Rueppell et al. (2005) found that age-specific mortality increased exponentially in drones after about the 10th day of flying activity, consistent with either senescence, non-replenishment of resources, or 'wear and tear'. They also reported that lifespan after the initiation of flying activity was negatively correlated with age at first flight, and suggested that this pattern was due to the onset of senescence even before the initiation of flight. This suggestion is consistent with our experimental results for workers.

Senescence of honey bee hemocytic cells has been reported by (Amdam et al., 2004) and (Amdam et al., 2005). Amdam et al. (2004) found that foragers had low zinc concentrations compared with nurses, which in turn resulted in decreased hemocyte counts in the hemolymph; foragers also possessed a higher number of pycnotic cells than nurses. Working with reverted nurses, Amdam et al. (2005) showed that these changes were related to both age and behavioral role; reverted nurses had a higher hemocyte count relative to similarly aged bees that continued to forage, but reverted nurses had lower counts relative to normal-age (young) nurses. The authors assumed that hemocyte count and cell pycnosis are measures of senescence at the cellular level. There are no data on the relationship between hemocyte count and immune response or mortality rate, so it is not clear in this case that cellular senescence leads to organismal senescence.

Our results show that worker bees show senescence. In contrast, in a recent study Rueppell et al. (2007) assessed age-dependent behavioral performance of foragers using a battery of behavioral tests that included light sensitivity, sucrose responsiveness, learning of olfactory cues, and walking velocity. In that study, the authors concluded that worker bees did not exhibit an age-dependent decline in performance but showed an increase in mortality with chronological age. The discrepancies between our results and those of Ruepell et al. may be attributed to the nature of the behavioral tests employed. Although the behavioral tests employed are related to foraging activity they may not prove demanding to the bees, and thus not allow the possibility for a decline to be manifest. Previous studies in *D. melanogaster* show that age-related declines in behavior differ, depending on the nature of the behavior being tested, the genotype, and the gender of the flies (Fernandez et al., 1999; Martin and Grotewiel, 2006; Simon et al., 2006). We have shown here that limited worker lifespan is due at least in part to intrinsic senescence and not solely to extrinsic mortality factors. Of interest would be to determine if honey bee queens also show senescence. Studying senescence in queens is a more difficult question to address than in workers, given their extended lifespan. In addition, conducting such tests in a eusocial species presents special challenges since queens are fed and groomed by workers. Although we did not directly study queen senescence, queens are known to lay up to 2000 eggs per day and the laying rate does not appear to decline at least through the first year of life (Winston, 1987), suggesting negligible senescence during this period. In contrast, we have shown that senescence in workers begins before 50 days of age. This comparison suggests that the extended lifespan of queens is due to slower senescence, and not just to lower extrinsic mortality.

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Table 1. Effects of starvation, heat, and hydrogen peroxide (oxidative stress) on lifespan for nurse honey bees 10, 30, and 50 days of age. Mean and median lifespan (h).

Treatment	Age class	Mean (S.E)	Median	Sample Size
Starvation	10	91 (5.0)	42	478
	30	46 (1.4)	36	375
	50	31 (0.9)	24	287
Heat	10	120 (2.2)	120	484
	30	107 (2.5)	102	371
	50	106 (2.6)	102	288
Hydrogen Peroxide	10	82 (2.5)	75	479
	30	81 (2.8)	66	371
	50	75 (2.5)	66	287

Table 2. Effects of starvation, heat, and hydrogen peroxide (peroxide) on lifespan for nurse honey bees 10, 30, and 50 days of age. Hazard ratios for each pairwise comparison between age classes (ratio of older to younger bees); degrees of freedom = 1 in every case. Results in bold indicate significant differences.

Treatment	Contrast	Hazard ratio	Confidence	limits	χ^2	P
Starvation	**10 vs. 30**	**1.514**	**1.280**	**1.791**	**23.4**	**<.0001**
Starvation	**10 vs. 50**	**2.637**	**2.183**	**3.184**	**101.4**	**<.0001**
Starvation	**30 vs. 50**	**1.741**	**1.469**	**2.064**	**40.9**	**<.0001**
Heat	**10 vs. 30**	**1.506**	**1.3**	**1.744**	**29.7**	**<.0001**
Heat	**10 vs. 50**	**1.739**	**1.475**	**2.049**	**43.5**	**<.0001**
Heat	30 vs. 50	1.155	0.982	1.358	3.0	0.08
Peroxide	10 vs. 30	1.073	0.927	1.241	0.9	0.34
Peroxide	**10 vs. 50**	**1.257**	**1.069**	**1.478**	**7.6**	**0.006**
Peroxide	30 vs. 50	1.172	0.998	1.376	3.7	0.05

Figure 1. Schematic of time-staggered experimental design, so that nurse bees of different age classes were available for treatment on the same day. Rows indicate each experimental (single-cohort) colony and columns indicate the collection dates for bees of the different age classes used in the three stress resistance tests. This experimental design enabled us to evaluate the effects of age on stress resistance, taking into account influences of both source colony and date of collection (seasonality). We collected 10-, 30-, and 50 day-old bees from all colonies with the exception of colony 4 (10- and 30-day-old bees only) and colony 5 (10-day-old bees).

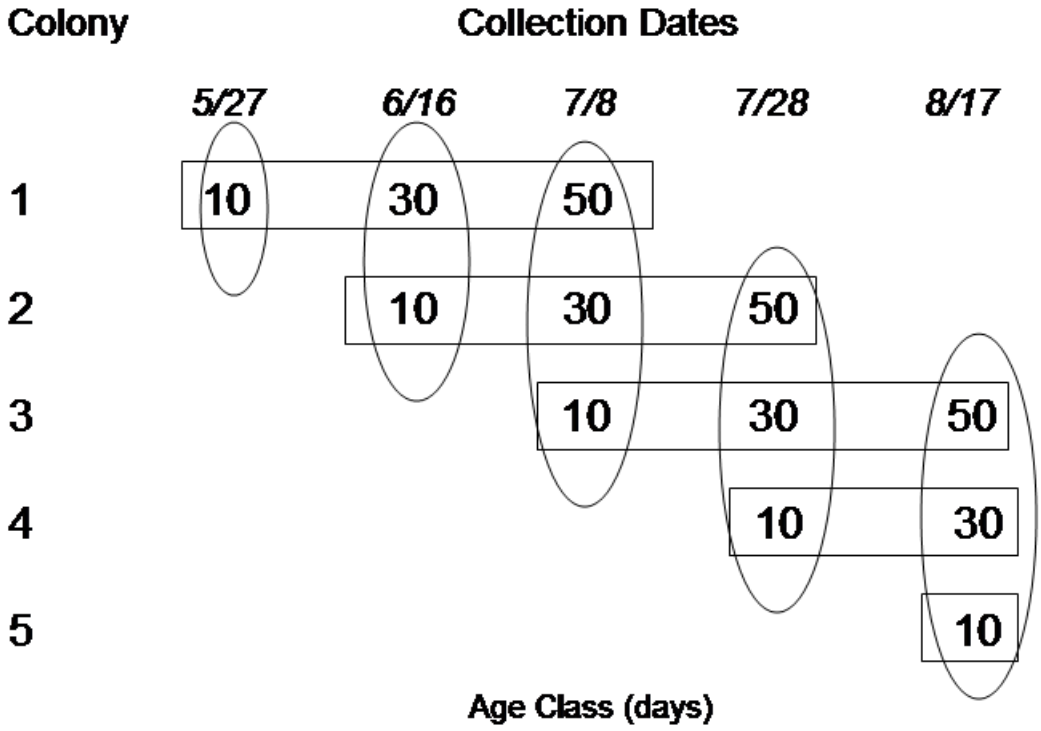
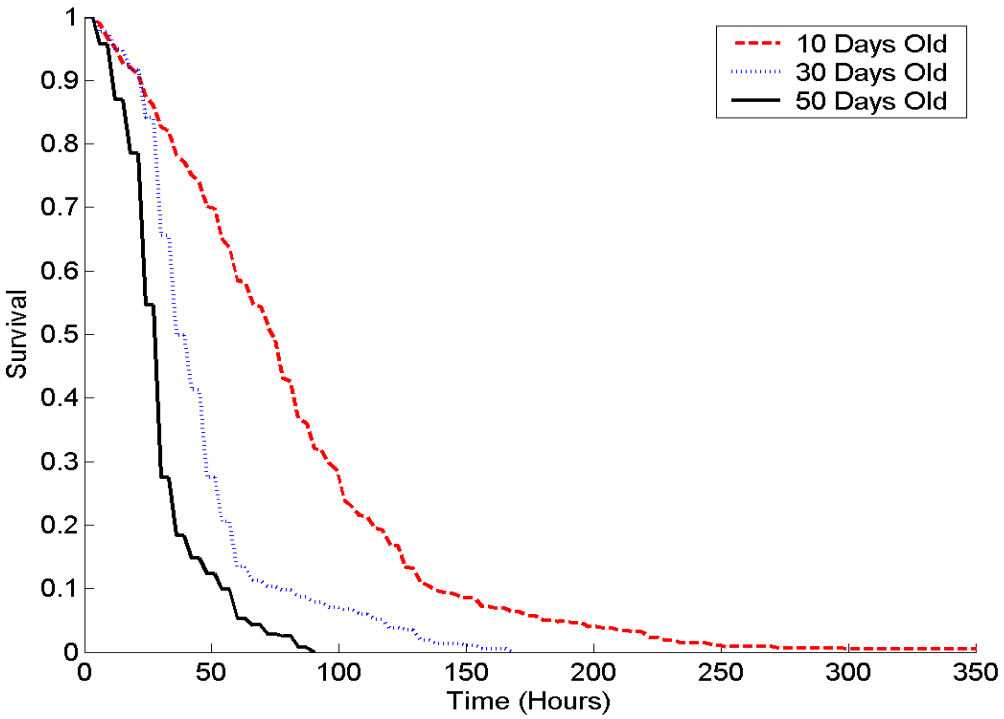


Figure 2. Age-related differences in resistance to: (a) starvation; (b) heat, and (c) hydrogen peroxide in nurse honey bees. Cumulative hazard function (-Log(Survival), bottom) and Survival distribution function (top) of 10- (red) 30- (blue) and 50-day-old (black) nurse bees. Bees were censused every 6 h. Note difference in scale for treatments. Eighteen 10-day-old bees were alive in the starvation treatment; one 10-day-old bee and one 30-day-old bee were alive in the hydrogen peroxide treatment after 350 h of exposure to stress.

2A Starvation



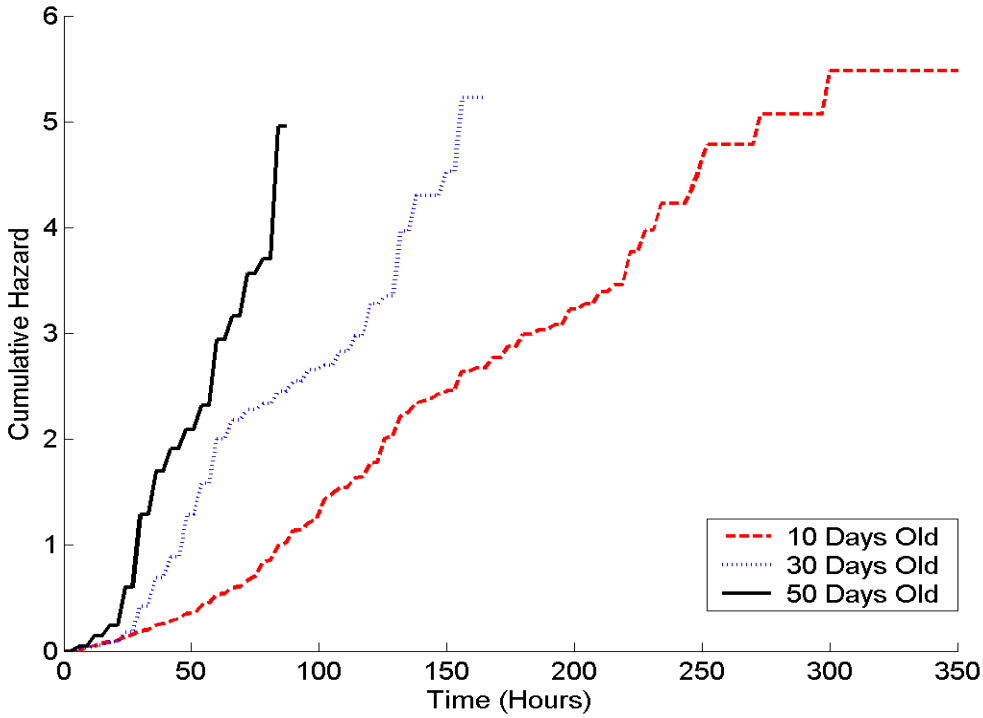
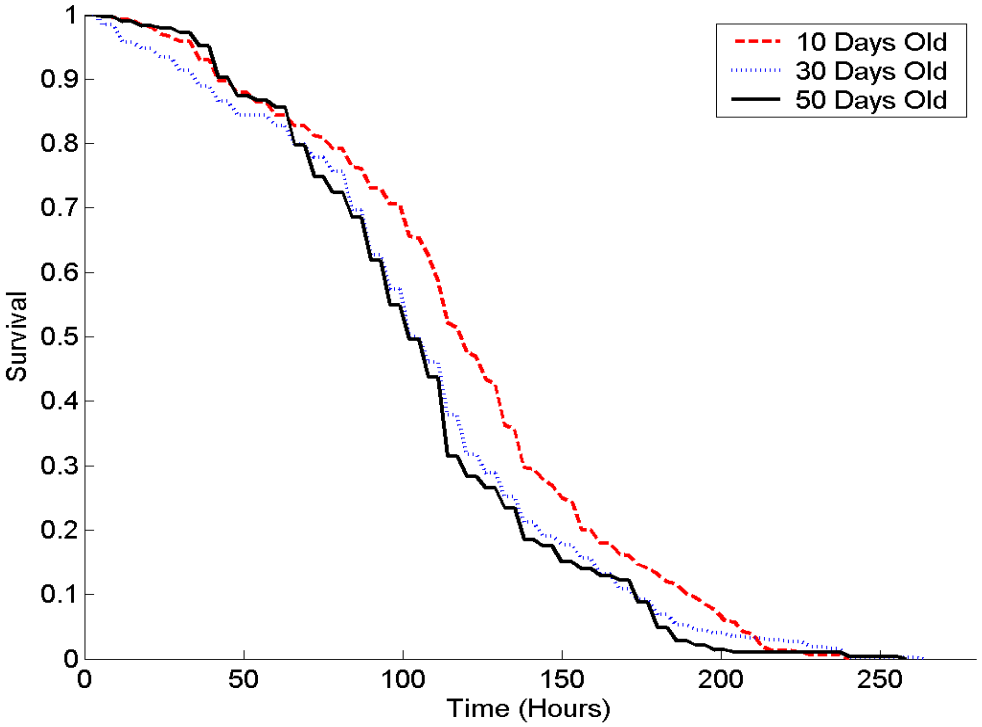


Figure 2 (cont.)
2B Heat



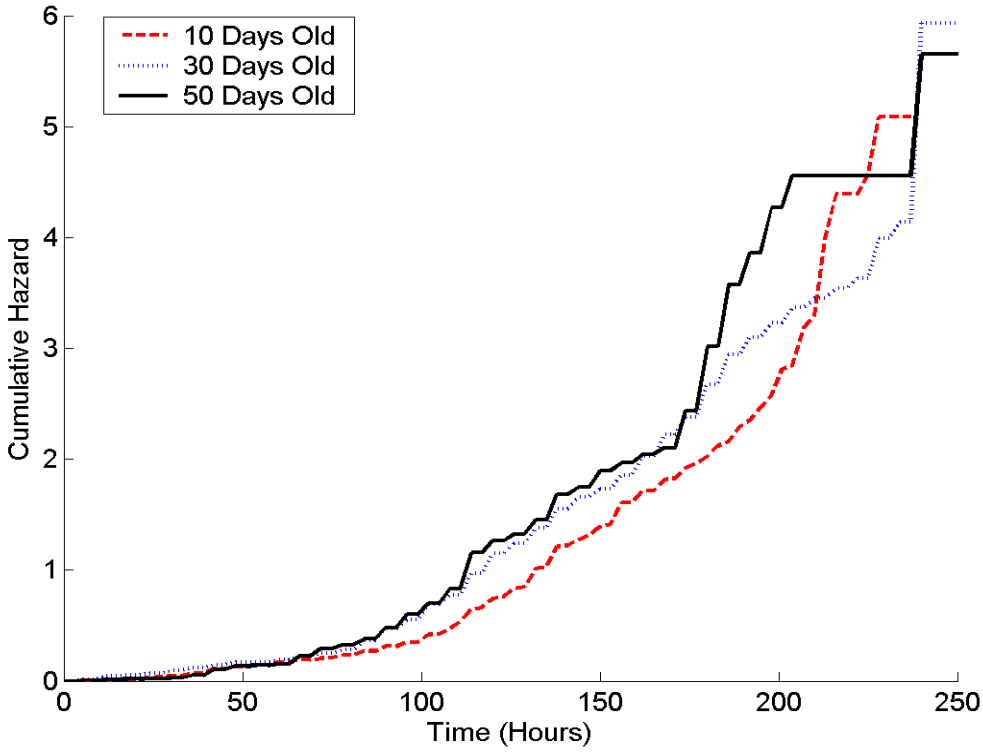
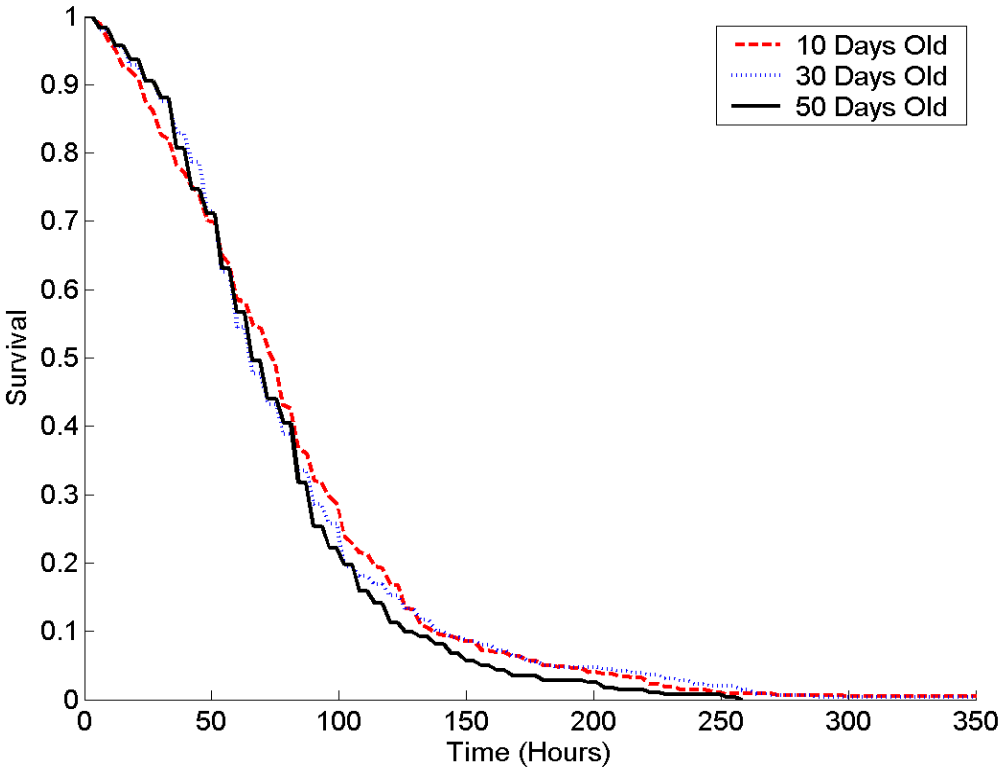


Figure 2 (cont.)
2C Hydrogen Peroxide



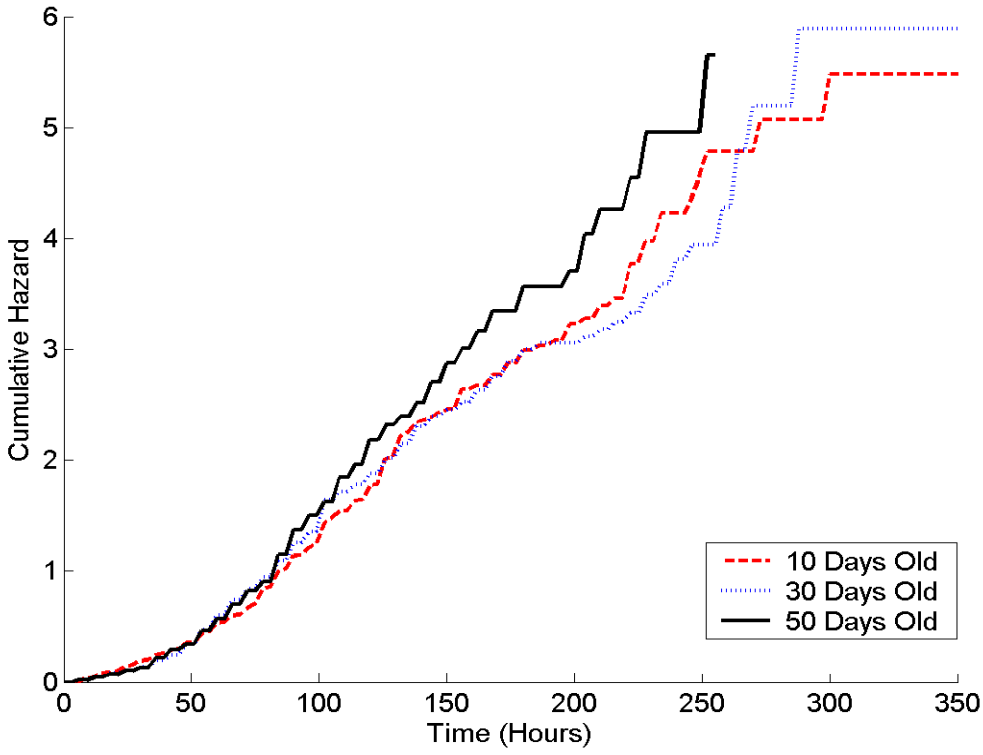
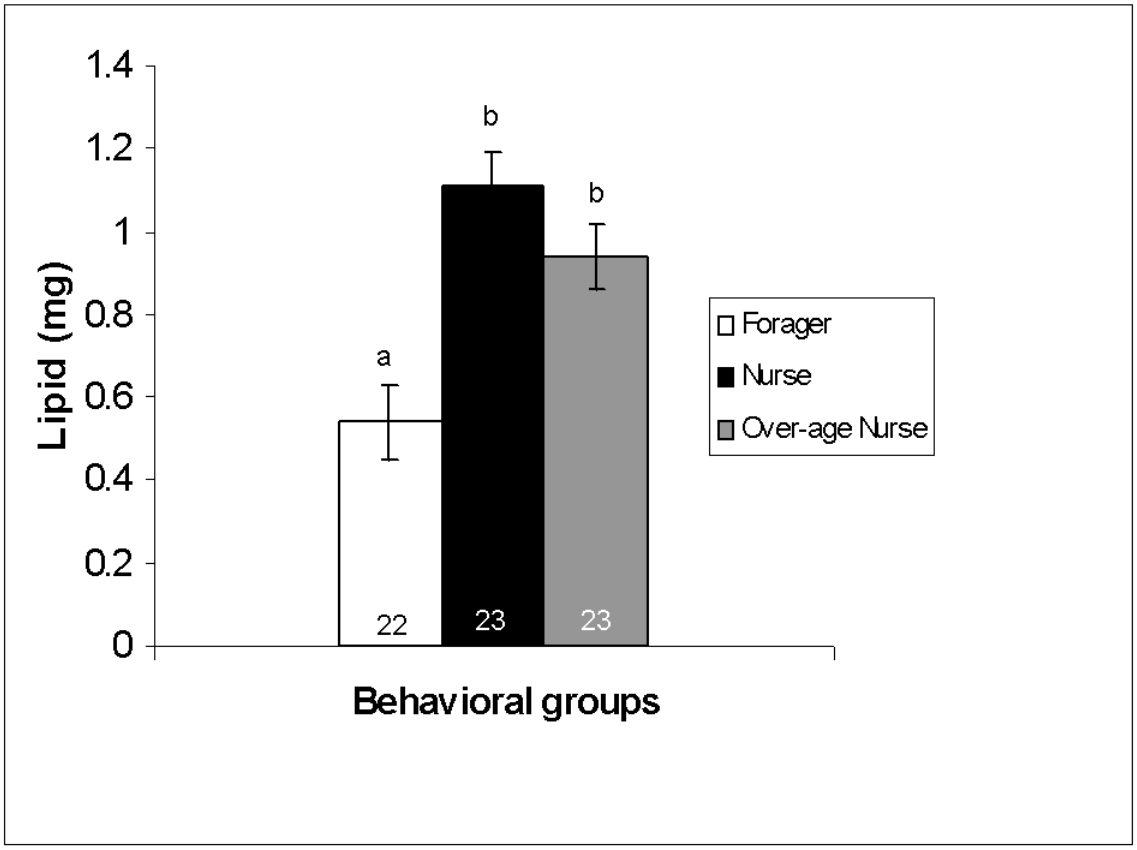


Figure 3. Abdominal lipid content for 7-day-old nurses, 50-day-old nurses, and 50-day-old foragers. Letters indicate groups that differ significantly in mean lipid content by pair-wise contrasts. Numbers at bottom of bars indicate sample size.



CHAPTER 2

ROLE OF THE INSULIN/INSULIN-LIKE SIGNALING PATHWAY (IIS) IN THE EVOLUTION OF LIFE HISTORY DIVERGENCE IN THE FRUIT FLY *D. melanogaster*

2.1. Introduction

The best-known example of a molecular mechanism that can regulate life history traits is a conserved signal transduction pathway involving insulin and insulin-like molecules. The Insulin/insulin-like signaling pathway (IIS) is a component of an evolutionarily conserved neurosecretory pathway that regulates development and aging in response to environmental cues such as food availability (Gems and Partridge, 2001). The IIS pathway is involved in the coordination of growth, metabolism, development, reproduction, and lifespan in *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals (Reviewed in Kenyon, 2001; Gems and Partridge, 2001; Taguchi and White, 2008). Genetic manipulation of the pathway can lead to lifespan extension in worms, flies and mice, and recent studies have linked genetic variation in components of the insulin-signaling pathway to long lifespan in humans (Van Heemst et al., 2005; Pawlikowska et al., 2009; Kojima, et al., 2004; Suh et al., 2008). The IIS pathway is hypothesized to be a mechanism used to tune the life history of an organism to the prevailing environment because it signals the availability of nutrients and it can potentially coordinate the allocation of energy between reproduction and body maintenance (Kenyon, 2001; Partridge and Gems, 2002; Gems and Partridge, 2001). Genes in this pathway are therefore obvious candidates for regulating life history patterns. Nevertheless, it has not been demonstrated that these genes actually do control natural variation in lifespan or reproduction in any organism.

Dissection of IIS pathway function first occurred in *C. elegans*. In the roundworm, the IIS pathway is characterized by a cell surface receptor (*daf-2*) that is activated by insulin-like

ligands. Thirty-seven such ligands have been identified in the nematode worm. The IIS signaling cascade ultimately deactivates a fork-head transcription factor (*daf-16*), which is inactivated by its translocation outside of the nucleus. The *daf-2* pathway also regulates the formation of a quiescent state know as the dauer which enables worms to survive adverse conditions such as food shortage or crowding. Worms in the dauer state are developmentally arrested, highly stress resistant, and non-reproductive. Once signaling through the *daf-2* pathway resumes, worms become reproductive and their lifespan is restored to that of the wildtype (Guarente and Kenyon, 2000). *Daf-2* regulates dauer formation and lifespan in a cell non-autonomous fashion (Apfeld and Kenyon, 1998). Secondary signals downstream of *daf-16*, hormonal signals in the germline and somatic gonad tissue also act to influence lifespan and reproduction (Hsin and Kenyon, 1999; Arantes-Oliveira et al., 2002).

A similar pathway operates in the fruit fly *D. melanogaster* (Figure 4). In flies, the insulin receptor (*InR*) transduces the signal from the insulin-like peptides (dilp's 1-7) to the phosphatidyl inositol-3-OH-kinase (*PI3K*) either directly or through the insulin receptor substrate (*chico*). *PI3K* phosphorylates phosphatidyl inositol-(4,5)-biphosphate (*PIP2*) to phosphatidyl inositol-(3,4,5)-triphosphate (*PIP3*). This activity is antagonized by the *PTEN* phosphatase. High levels of *PIP3* recruit *PKB* to the plasma membrane where it can be further activated by the phosphoinositide-dependent kinase (*PKD*). Activated *PKB* levels are associated with the phosphorylation and inactivation of the fly forkhead transcription factor (*FOXO*) and its exclusion from the nucleus. Elevation of IIS signaling results in decreased *FOXO* transcriptional activity in the nucleus and decreased IIS signaling leads to increased transcriptional regulation by *FOXO* (Giannakou and Partridge, 2005). *FOXO* serves as a transcription factor that can up-regulate genes involved in lifespan extension, such as antioxidant and detoxification genes.

Interventions known to increase lifespan in fruit flies include mutations in the insulin receptor (*InR*) (Tatar et al., 2001), insulin receptor substrate (*chico*) (Clancy et al., 2001), over-expression of *FOXO* in the head and abdomen fat body (Hwangbo et al., 2004, Giannakou et al., 2004), over-expression of *PTEN* in head fat body (Hwangbo et al., 2004), and decreased expression of insulin-like ligands (dilp's) (Broughton et al., 2005). The phenotypes seen in long-lived *Drosophila* with decreased IIS signaling are similar to those seen in long-lived worms: reduced or delayed fecundity, increased stress resistance, and increased fat storage (Giannakou and Partridge, 2007).

In flies, as in worms, lifespan and reproduction are coordinated by endocrine signals. Insects produce a sequisterpenoid hormone known as juvenile hormone (JH). JH is involved in complex processes such as development, reproduction, diapause, migration, and division of labor in social insects. During reproduction, JH regulates germline maturation, vitellogenesis, courtship behavior, and pheromone production (Klowden, 2002). InR and Chico mutants have reduced JH synthesis (Tu et al, 2005). Addition of JH to diapausing flies restores vitellogenesis and shortens lifespan (Tatar et al, 2001). Other hormones involved in metamorphosis and reproduction are ecdysteroids. InR mutants show reduced synthesis of ecdysteroids in the ovary (Tu et al, 2002). Mutations on the ecdysone receptor (EcR) have been shown to increase lifespan in flies (Simon et al, 2003). From these observations, JH and ecdysteroids have been hypothesized to serve as secondary pro aging signals downstream of the IIS pathway. JH and ecdysteroids promote reproduction and shorten lifespan (Tu et al, 2006).

To determine whether genes in the IIS pathway are responsible for natural variation in lifespan and reproduction, I combined experimental evolution and gene expression analysis. First, I created strains of flies that differ in their life histories and show a genetically based

tradeoff between early and late life performance. I then used quantitative real-time PCR (qRT-PCR) to measure mRNA expression of five genes in the IIS pathway (*dilp2*, *InR*, *chico*, *pkb, foxo)* that have been shown to cause lifespan extension in *D. melanogaster*.

In addition, I measured mRNA expression of two yolk protein genes. I included the yolk protein genes YP1 and CG31150 because recent experiments have implicated a yolk protein in the extended longevity and high late-life fertility of queen honey bees. In honey bees, the protein vitellogenin (*Vg*) is involved in yolk deposition in eggs, and has other pleiotropic functions. It regulates division of labor and serves as an antioxidant that scavenges free radicals that cause oxidative stress (Seehuus et al., 2006; Munch et al., 2008). Long-lived and highly fertile queen honey bees have higher vitellogenin titers in the head, thorax, and abdomen than female honey bees of the worker caste (Corona et al., 2007). To test the hypothesis that evolution of long life and high late-life fertility in my selection lines exploited the same mechanism that has been postulated to confer these traits in social insect queens (which have the longest-lived adult stages known in insects), I evaluated the fly ortholog of honey bee *Vg* (CG31150) and *YP1*, which is the most abundant yolk protein in female fruit flies.

2.2. Methods

2.2.1. Selection on Life History

To study the effects of the IIS pathway on life-history divergence I created 3 paired and replicated fly lines with divergent life histories: flies in the Control (C) lines have increased early fecundity and a short lifespan, whereas flies in the Selection (S) lines have high late life fecundity and increased lifespan. The S and C lines within a pair were initiated from the same 320 male and 320 female flies, so that the lines started with the same genetic composition. The

three different S-C pairs were started from three different sets of parents to create independent draws from the same ancestral population. The ancestral population was derived from approximately 8000 offspring of 400 wild caught females collected in New Jersey in 1998 and maintained in the lab at large population size (>5000 individuals) with overlapping generations. Flies in the ancestral population were transferred to new media every 2 weeks.

Each generation, S and C lines were propagated from 320 single-pair matings. Equal numbers of virgin offspring were collected from each pair and allowed to age in single sex vials at a density of 25 flies per vial. In S lines, only flies that survive and are fertile at >44 days of age can contribute to the next generation, while in C lines, flies that survive and are fertile at 14 days will contribute to the next generation. C lines were thus maintained in a similar fashion compared to flies in the ancestral population. To avoid differential selection on developmental time, we collected newly eclosed virgin adults at a fixed time after egg-laying (10-12 days post-mating).

After virgin flies from the S and C lines reached the appropriate age, single virgin females and virgin males were paired to produce 320 single pair matings. Flies were paired at random to minimize inbreeding. Only flies that survived and were fertile at the time of mating were able to contribute to the next generation (C lines: 14 days of age, S lines: 44 days of age).

2.2.2. Lifespan Assays

We collected 1,600 virgin female flies from each control and selection line to assay adult survival. Flies were lightly anesthetized using CO_2 and 6 female flies were placed into 8-dram vials with cornmeal media. We established 100 vials each with 6 virgin females per S and C line. Flies were kept in an incubator at 25C on a 12L:12D light cycle. Flies were transferred to fresh

media every week. At the time of transfer I recorded the number of flies that were alive. Flies that escaped the vial at the time of transfer were recorded as censored.

2.2.3. Age-specific Fecundity Assay

Fecundity was measured at 7, 14, 35, 44, and 60 days of age for all S and C lines. For every assay, three virgin females and three virgin males of the appropriate age class were allowed to mate in a single vial. Trials for every age class included fifty vials each with three mating pairs of flies. Flies were allowed to mate for twenty-four hours and were discarded after this time period. Before discarding flies, I recorded the number of females that were alive. Offspring were allowed to develop to adulthood and after 17 days of the initial mating between the parents; offspring that had emerged were frozen and counted.

2.2.4. Age-specific Gene Expression

To determine if S and C lines differed in a consistent way with respect to expression of IIS genes and yolk protein genes, I collected newly-emerged female flies from two S-C pairs and allowed them to age for 14, 44, and 60 Days. C flies were collected and frozen at 14 and 44 days of age; S flies were collected and frozen at 14, 44, and 60 days of age. Flies were transferred to fresh media every week. Once flies reached the appropriate age, they were lightly anesthetized using CO_2 and frozen on dry ice. All flies were frozen at 9 a.m. to avoid circadian effects on gene expression. For each line-age-tissue combination, I assessed gene expression in 10 independent (biological) replicates, and pooled tissue from 10 individual females for each replicate. Flies were stored at -80C for later dissection. I extracted RNA from pooled heads and abdomens using TRIzol (Invitrogen) and the manufacturer's protocol. RNA was quantified using a spectrophotometer (Nanodrop) at 260 nm, and checked for purity by examining the 260/280

nm ratio. All samples had a ratio > 1.8. RNA samples were treated with Turbo DNAse (Ambion) to eliminate any remaining genomic DNA following the manufacturer's instructions.

For cDNA synthesis, 200 ng of RNA were reverse transcribed using a mixture of 2μl 10X first strand ArrayScript buffer (Ambion), 1μl 10mM dNTP mix (Applied Biosystems), 0.2 μl RNase inhibitor (Applied Biosystems), and 0.2 μl 200U/ul ArrayScript (Ambion). I spiked in 0.1μl of RCP1 (root cap protein) cRNA into each reaction as an exogenous control. Gene quantity values of each sample were normalized to corresponding RCP1 quantity values to account for variation in cDNA synthesis reactions. Reactions were incubated at 42ºC for 60 minutes then at 95ºC for 5 minutes.

I used quantitative real-time polymerase chain reaction (qRT-PCR) to measure mRNA abundance levels of *dilp2*, *InR*, *chico*, *pkb, foxo*, fly *yolk protein*, and *CG31150* in two body segments (head and abdomen). *dilp2* mRNA abundance levels were assayed only in head tissue because this ligand is produced in insulin producing cells (IPC's) in the brain and is not expressed in the abdomen. I assayed separate tissues (head and abdomen) because expression profiles can vary between tissues (Girardot et al., 2006; Zhan et al., 2007) and also because levels of *dilp2* and *foxo* in the fly head can serve as global regulators of IIS signaling in other tissues of the body (Hwangbo et al., 2004). Different physiological activities occur in head and abdomen tissue. For example, nutrient absorption and reproductive activities can have an influence on gene expression profiles of abdomen tissue.

qRT-PCR was performed with a TaqMan 7900 Cycler, using SYBR Green Master Mix (Applied Biosystems). I designed forward and reverse primer sets for each of the 7 genes using Primer Express software v2.0 (Applied Biosystems) (Table 3). I checked primer sets for specificity using NCBI BLAST to insure that they matched only the target gene and that they

would not amplify another region of the *Drosophila* genome. I quantified the amount of initial mRNA by using the standard-curve absolute-quantification method. A standard curve was generated for each gene using serial dilutions of *Drosophila* genomic DNA (0.001 ng, 0.01 ng, 0.1 ng, 1.0 ng, and 10.0 ng). I quantified the initial amount of mRNA for RCP1 in each sample using a standard curve made of serial dilutions of RCP1 cDNA. The starting amount of mRNA from each sample was determined using the appropriate standard curve for each gene. qRT-PCR reactions were conducted using the default PCR cycle settings for 40 cycles. A dissociation curve was added to the final cycle to confirm the absence of primer-dimers for each gene.

2.2.5. Data Analysis.

Statistical analysis was conducted using SAS software, version 9.2 of the SAS system for Windows (Copyright, SAS Institute Inc). Survival data was analyzed using SAS Proc Lifetest. This is a nonparametric test that estimates the survival function. Kaplan-Meier survival estimates were used to graph the percent survival versus time in weeks. I conducted two rank tests, Log-rank and Wilcoxon, which test for homogeneity of survival functions between S and C lines. The log-rank test places more weight on larger survival times and the Wilcoxon test places more weight on shorter survival times. Log-rank and Wilcoxon P-values were the same for both analyses; therefore, I only reported the log-rank test values in the results.

Fecundity data was analyzed using the Wilcoxon-Mann-Whitney test, a non-parametric version of the t-test. The mean number of offspring per female was the dependent variable. Comparisons were made between S and C lines for each replicate at each age. qRT-PCR data was analyzed using parametric analyses because the data were normally distributed and approximately homoscedastic. Head and abdomen tissues were analyzed separately for each gene. Expression values with studentized residuals greater than 2 were considered outliers and

deleted at the level of technical replicates. I calculated mean expression values at the biological replicate level and deleted outliers using the same criterion. Data analysis was conducted separately for each of the S-C pairs. Table 4 shows the number of biological replicates included in the final analysis. To account for differences in cDNA synthesis, I normalized the remaining quantity values by dividing each mean quantity value by its corresponding mean RCP quantity value. I used SAS PROC MIXED to fit the model: $y = \mu + B + A + L + A*L + e$ where y is RNA expression level, B is the random effect of cDNA synthesis batch, A is the fixed effect of age, L is the fixed effect of line, and e is the residual error. Least-square means and standard errors were used to produce bar graphs of the normalized expression quantity versus each age and line.

2.3. Results

2.3.1. Lifespan and Age-specific Fecundity

All S-C pairs diverged significantly in lifespan in the expected direction (S>C: $\chi 2$ 64.12, d.f. 1, P<0.0001). Flies in the selection lines lived longer than flies in the control lines (Fig 5). Early life fecundity was higher for C line females than for S line females (Kruskal-Wallis, p<0.0001 for Set 1S; p<0.0001 for Set 2S; and p <0.0183 for Set 3S). In lines 1 and 3 this difference was observed at 7 days of age, in line 2 it was observed at 14 days of age. Late-life fecundity was higher for S line females than for C line females in all lines (Kruskal-Wallis, all p<0.0001 for all S-C comparisons on Day 44) (Table 5, Figure 6).

2.3.2.Age-specific Gene Expression

There were no significant differences in IIS gene expression between S and C lines for either the head or abdomen tissues (Table 6 reports P values for each test of the model for each

gene). These data suggest that differential gene expression in the IIS pathway is not involved in the regulation of divergent life-histories between the S and C lines. Age-related changes in gene expression showed variation between fly sets in both head and abdomen tissue. Overall there were more significant changes in gene expression with age for set 2 than set 3 and these changes might have been more pronounced in set 2 because there is a sharp decrease in gene expression with age when comparing younger to older flies whereas there seems to be little change in expression patterns with age in set 3. This general trend was observed for both head and abdomen tissue (Figure 7 and Figure 8).

2.4. Discussion

I successfully generated fly lines that show a genetically based tradeoff between lifespan and reproduction. Flies in the selection line live longer than flies in the control line. Furthermore, flies in the selection line show reduced early life fecundity and increased late life fecundity whereas the opposite pattern is true for flies in the control line. My main goal in this study was to investigate the involvement of genes in the IIS pathway in the evolution of divergent life histories observed in our fly lines. Our working hypothesis was that flies selected for increased lifespan and late-life fecundity would exhibit lower IIS signaling. However, our gene expression data do not support a role for the IIS pathway in lifespan extension of our fly lines.

Flies in the S lines were expected to show low expression of the genes *dilp2*, *InR*, *chico*, and *PKB* and higher levels of *foxo* early in life compared to C lines, because resource allocation is devoted to somatic maintenance and not to reproduction. Levels of these genes would increase later in life in S lines once resources are shifted to reproduction. Higher levels of *foxo* expression in the S lines would suggest that this transcription factor would up-regulate the expression of

longevity-enhancing genes such as oxidative stress resistance genes and detoxification genes (Murphy et al., 2003) allowing the S lines to live longer and have higher fecundity late in life.

Variation in the IIS pathway, particularly at the *InR* locus, has been implicated as a cause for variation in lifespan in natural populations of *Drosophila*. Geiger-Thornsberry and Mackay (2004) used quantitative complementation tests to identify genes that contribute to naturally occurring variation in *Drosophila* lifespan. One of the genes they found to affect variation in lifespan using this method was the *InR* gene. A recent study by Paaby et al. (2010) identified polymorphisms at the *InR* locus that are associated with variation in life history patterns observed in natural populations of flies that show a latitudinal cline both in North America and Australia. The authors also found evidence of positive selection on the InR locus. It is possible that changes to the IIS pathway are involved in differences observed in S vs. C lines, but that those changes are not reflected in measures of mRNA abundance.

In a previous study, Williams et al. (2006) used two natural *D. melanogaster* diapause variants from Canada and the Southern U.S. to identify genes involved in the regulation of diapause. The authors were able to map genes involved in the variant diapause phenotypes to chromosome III and identified *PI3K* as a candidate gene for diapause control in flies. Over-expression of this gene in fly heads resulted in a significant reduction in diapause leading the authors to measure differences in gene expression in fly heads and bodies. Findings from this study showed no differences in *PI3K* gene expression between the diapause variants. However, the variants showed differences in DNA sequence within non-coding regions of the gene pointing to the involvement of *PI3K* in diapause regulation through a mechanism other than differential gene expression. Post-transcriptional changes to genes involved in the IIS pathway may regulate lifespan differences. There might be consistent differences in other regulatory

mechanisms (phosphorylation or glycation of proteins) that could influence lifespan and reproduction, which would not be revealed by qRT-PCR analysis.

An alternative explanation to our results is that variation in other signaling pathways or at particular loci is responsible for the evolution of divergent life histories in our fly lines. Several studies have uncovered genes that increase lifespan in fruit flies including stress resistance genes such as heat shock proteins, and antioxidants such as MnSOD (Sun and Tower, 1999; Ruan et. al., 2002; Chavous et. al., 2001; Arking, 1998; Arking et. al., 2000; Arking, 2001; Mockett et. al., 1999; Orr and Sohal, 1994; Khazaeli et. al., 1997; Orr and Sohal, 1993). Histone deacetylases such as *Sir2* and *Rpd3* have been implicated in yeast lifespan extension although their role in lifespan extension in fruit flies is not well understood (Chang and Min, 2002; Kaeberlein et. al., 1999; Kim et. al., 1999; Guarente, 2000; Rogina et. el., 2002). Mutation screens and QTL studies have identified genes that extend lifespan such as the membrane protein Methuselah (*mth*) (Lin et al., 1998), dopa decarboxylase (*Ddc*) and catecholamines up (*catsup*) (De Luca et al., 2003; Carbone et al., 2006). An alternative approach to uncover the molecular mechanisms involved in mediating life history divergence in our fly lines is the use of unbiased genomic profiles to assess sequence and/or expression changes that are consistently associated with life history evolution. This will allow us to uncover previously characterized/novel genes that could be implicated in regulating tradeoffs between lifespan and reproduction in our fly lines.

Another goal of this study was to explore the involvement of yolk proteins in extending lifespan in *Drosophila*. The yolk protein vitellogenin acts as an antioxidant in honey bees (Seehuus et al., 2006; Munch et al., 2008). Long-lived queen honey bees have higher titers of this protein than sterile workers and they can tolerate oxidative stress better (Corona et. al., 2007). We evaluated the expression of *YP1* and the honey bee *Vg* ortholog *CG31150* in our fly

lines. We did not find differences in gene expression between the S and C lines suggesting that the *Vg* ortholog *CG31150* is not involved in the lifespan differences observed for our lines. It is possible that the honey bee *Vg* ortholog plays a role in antioxidant stress resistance in flies, but in order to establish this one would need to evaluate oxidative stress resistance in flies with normal and increased levels of gene expression. Testing the role of honey bee *Vg* as an antioxidant would require the use of transgenic constructs in order to manipulate expression of this gene in flies exposed to oxidative stress resistance. Overall, I did not find significant differences in transcript abundance of genes involved in IIS signaling or yolk proteins involved in antioxidant resistance in honey bees to influence the contrasting patterns of lifespan and reproduction observed in S vs. C lines.

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Table 3. Primers designed for qRT-PCR

Gene	Forward Primer	Reverse Primer
Dilp-2	5'-AAGCCTTTGTCCTTCATCTCGAT-3'	5'-CCTTGGGCCAACTTCACTGT-3'
Inr	5'-TGCCGGTTATTTAATAAAGCTCAAC-3'	5'-CGTATCCCGCTATTGAATTGG-3'
Chico	5'-CCTAACTCTGCACGCCACAA-3'	5'-GGCTTCATCTCGAGGTAACCAT-3'
Pkb	5'-CAAGCCATTCACCTTCATCATC-3'	5'-CGGCAAATGTCCTTTCGATT-3'
Foxo	5'-GGTCAACACGAACCTGGTCAA-3'	5'-GCCGGAATTGCTGCTTATGT-3'
YP1	5'-CCAAAGCGGCGACATCAT-3'	5'-ATGTCGAGCATGGCATAACG-3'
CG31150	5'-CGCTGCTGGTGGCTGACT-3'	5'-ACAGGCGAAGGCGTAACAAA-3'
RP49	5'-CCCACCGGATTCAAGAAGTTC-3'	5'GGATGAGCAGGACCTCCAG-3'
RCP	5'-TCAATTAACTCGGAATCGGA-3'	5'-CCTGGATTTCCCTGCTGAT-3'

Table 4. Number of biological replicates used in qRT-PCR analysis for abdomen and head tissue

Set 2					**Set 3**				
Gene	Line	Age	Abd	Head	Gene	Line	Age	Abd	Head
Dilp-2	C	14		8	Dilp-2	C	14		8
	C	44		8		C	44		10
	S	14		9		S	14		9
	S	44		10		S	44		9
	S	60		8		S	60		10
Inr	C	14	9	7	Inr	C	14	10	7
	C	44	9	7		C	44	9	7
	S	14	10	8		S	14	9	8
	S	44	9	9		S	44	10	7
	S	60	10	8		S	60	9	8
Chico	C	14	8	6	Chico	C	14	9	8
	C	44	10	7		C	44	9	9
	S	14	9	8		S	14	7	8
	S	44	9	10		S	44	10	8
	S	60	9	9		S	60	9	8
PKB	C	14	9	7	PKB	C	14	9	7
	C	44	9	7		C	44	9	7
	S	14	10	8		S	14	8	8
	S	44	9	7		S	44	10	8
	S	60	10	8		S	60	9	8
Foxo	C	14	9	6	Foxo	C	14	9	6
	C	44	10	7		C	44	9	7
	S	14	9	7		S	14	9	9
	S	44	9	8		S	44	9	8
	S	60	10	8		S	60	9	7
CG31150	C	14	9	8	CG31150	C	14	10	6
	C	44	10	7		C	44	9	7
	S	14	9	8		S	14	8	9
	S	44	9	10		S	44	10	8
	S	60	10	9		S	60	9	8
YP1	C	14	9	6	YP1	C	14	9	7
	C	44	10	7		C	44	8	8
	S	14	9	8		S	14	8	8
	S	44	9	10		S	44	10	8
	S	60	9	9		S	60	9	9

Table 5. Mean number of offspring per female and Wilcoxon p-values for flies in the control (C) and selection lines (S).

Set/Line	Age	N	Mean number of offspring per female	Wilcoxon-Mann-Whitney Z (df=1)	p-value
1C	7	50	20.77 (0.98)	7.1053	<.0001
1S	7	50	9.0067 (0.71)		
1C	14	50	15.594 (0.65)	0.4414	0.6589
1S	14	50	14.9788 (0.74)		
1C	35	50	7.3674 (0.88)	0.2276	0.8199
1S	35	50	5.948 (0.54)		
1C	44	50	0	-8.9212	<.0001
1S	44	50	5.8033 (0.61)		
1C	60	34	0	-2.7463	0.006
1S	60	50	0.516 (0.18)		
2C	7	50	10.6527 (0.84)	7.46	<.0001
2S	7	45	24.6956 (1.01)		
2C	14	49	18.8422 (1.23)	5.44	<.0001
2S	14	50	9.9067 (0.85)		
2C	35	50	10.0233 (0.74)	-1.98	0.048
2S	35	50	13.362 (1.14)		
2C	44	50	5.688 (0.5)	-4.01	<.0001
2S	44	50	10.0247 (0.75)		
2C	60	13	0.1795 (0.12)	1.16	0.24
2S	60	26	0.1346 (0.13)		
3C	7	50	24.6133 (1.26)	-2.35	0.02
3S	7	49	20.1633 (1.12)		
3C	14	50	10.3713 (0.6)	-2.63	0.009
3S	14	50	13.7167 (1.0)		
3C	35	50	1.5167 (0.32)	-4.74	<.0001
3S	35	50	5.6933 (0.7)		
3C	44	50	0.3067 (0.13)	-7.49	<.0001
3S	44	50	3.1433 (0.35)		
3C	60	50	0 (0)	-3.3	0.001
3S	60	50	0.33 (0.12)		

Table 6. Effects of life history divergence on age-specific gene expression. Results are shown for abdomen and head tissue separately.

Abdomen Tissue

Gene	Set	Age		Line		Age*Line	
		F-Value	P-value	F-Value	P-value	F-Value	P-value
CG31150	2	1.439	0.249	0.202	0.655	0.048	0.826
	3	8.091	0.001	1.266	0.267	0.089	0.766
Chico	2	7.423	0.001	0.050	0.823	0.611	0.438
	3	2.769	0.076	1.623	0.210	0.999	0.324
FOXO	2	7.519	0.001	0.564	0.456	0.020	0.887
	3	0.677	0.514	0.364	0.549	1.309	0.259
INR	2	3.076	0.057	0.146	0.703	0.011	0.915
	3	13.913	3.1208E-05	0.180	0.673	0.036	0.848
PKB	2	10.308	0.0002	0.019	0.889	0.808	0.374
	3	1.458	0.245	0.937	0.339	2.769	0.104
YP1	2	3.188	0.0521	0.431	0.515	0.293	0.590
	3	7.102	0.0024	3.707	0.061	0.002	0.959

Head Tissue

Gene	Set	Age		Line		Age*Line	
		F-Value	P-value	F-Value	P-value	F-Value	P-value
CG31150	2	6.589	0.003	2.045	0.160	2.478	0.123
	3	1.897	0.163	1.101	0.300	0.129	0.720
Chico	2	5.230	0.009	4.283	0.045	5.282	0.027
	3	1.047	0.360	1.429	0.239	0.827	0.368
DILP2	2	9.981	0.0003	0.643	0.427	1.590	0.215
	3	4.358	0.019	0.405	0.527	0.055	0.814
FOXO	2	7.206	0.002	2.575	0.116	2.473	0.124
	3	3.704	0.034	0.907	0.346	0.091	0.763
INR	2	4.089	0.025	2.211	0.145	1.957	0.170
	3	1.527	0.229	1.269	0.266	2.065	0.158
PKB	2	8.444	0.0009	1.759	0.193	1.580	0.216
	3	0.767	0.470	1.570	0.217	1.019	0.318
YP1	2	7.696	0.001	1.314	0.258	4.129	0.048
	3	0.810	0.451	0.141	0.708	0.772	0.384

Figure 4. Insulin signaling pathway (IIS) in *Drosophila melanogaster*.

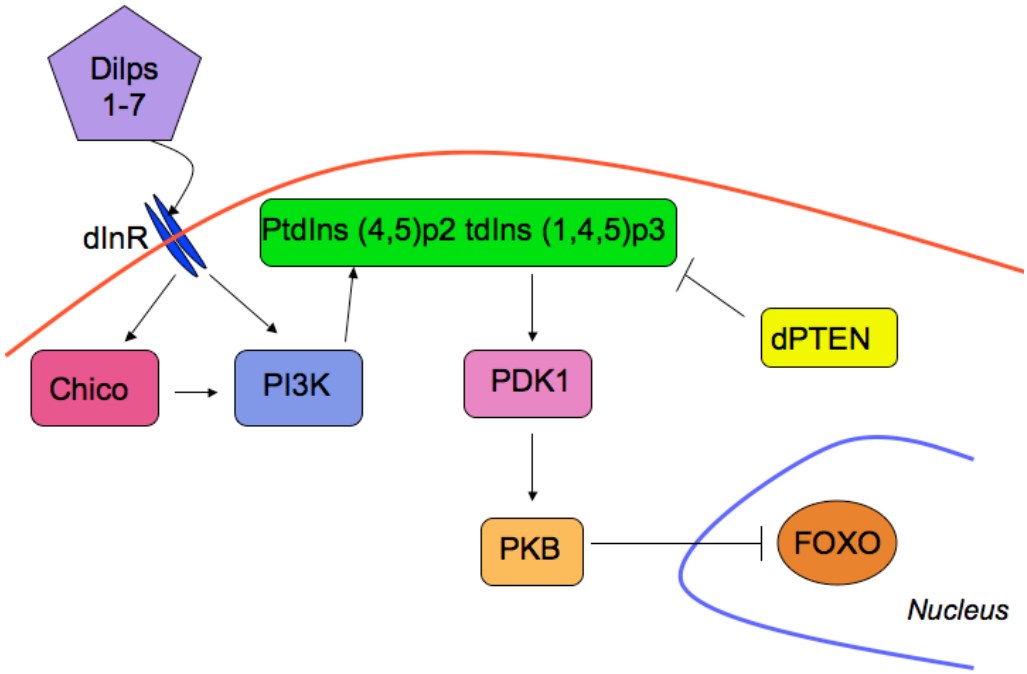


Figure 5. Survival curves for flies in the Selection (S) and Control (C) lines. S lines are shown in solid lines and C lines in dashed lines. Survival curves are shown in blue for set 1, black for set 2 and red for set 3.

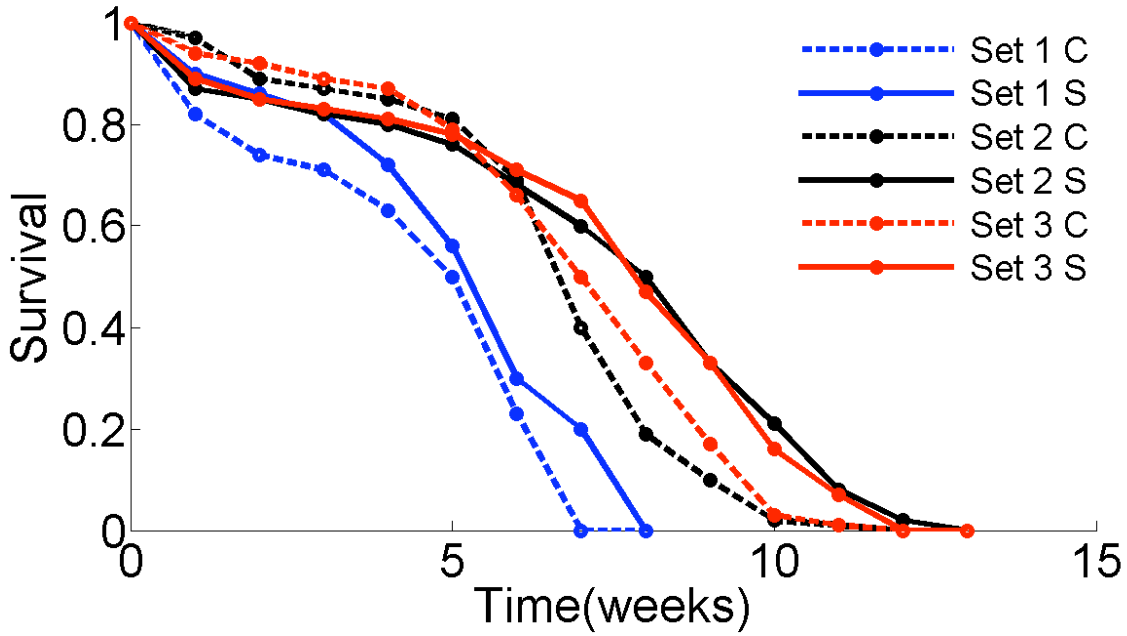


Figure 6. Mean adult offspring number for flies in the Selection (S) and Control (C) lines. Results are shown in blue for set 1, black for set 2 and red for set 3.

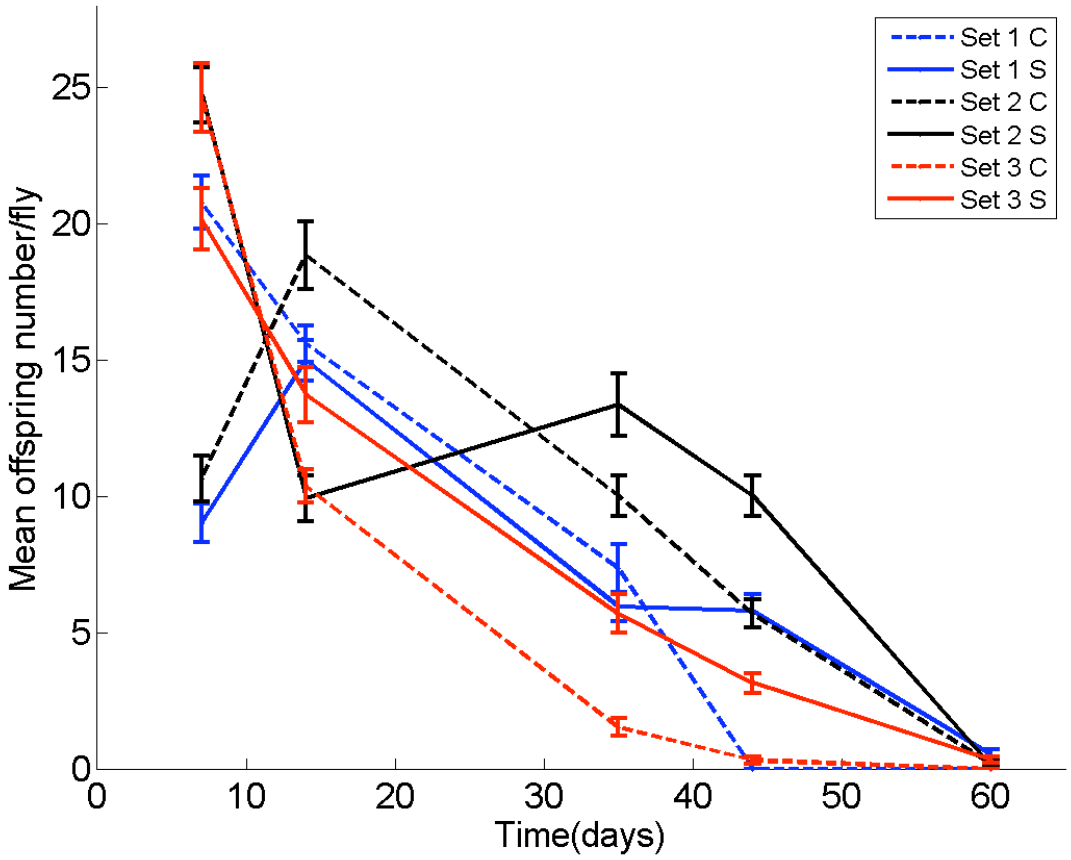


Figure 7. Relative mRNA expression levels in fly head tissue. Letters indicate RNA expression levels for genes in the IIS pathway and vitellogenins (*YP1* and *CG31150*). mRNA levels are shown on the y-axis and Line/Age combinations are shown on the x-axis. Expression bars for set 2 fly lines are coded in blue and set 3 fly lines in grey.

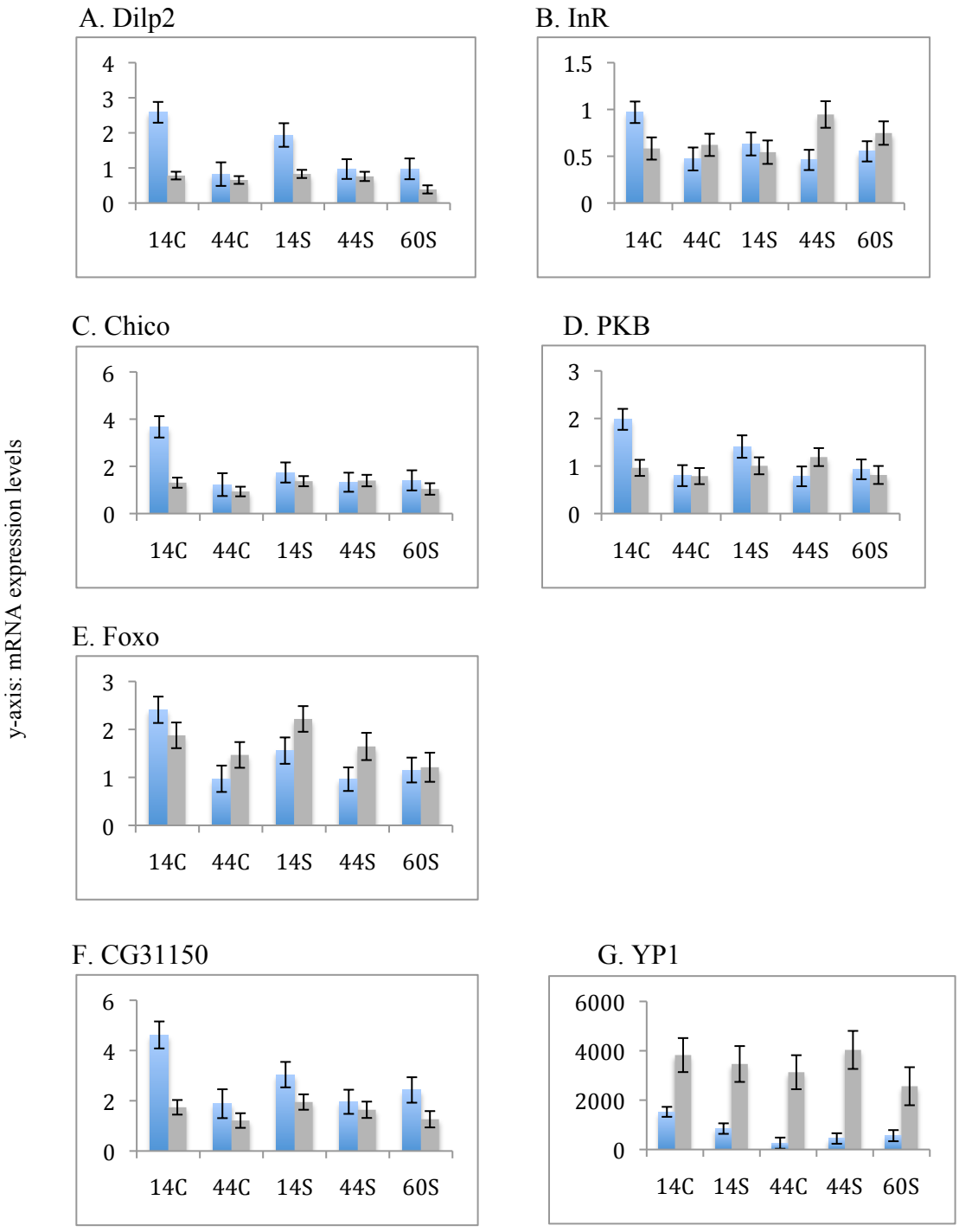
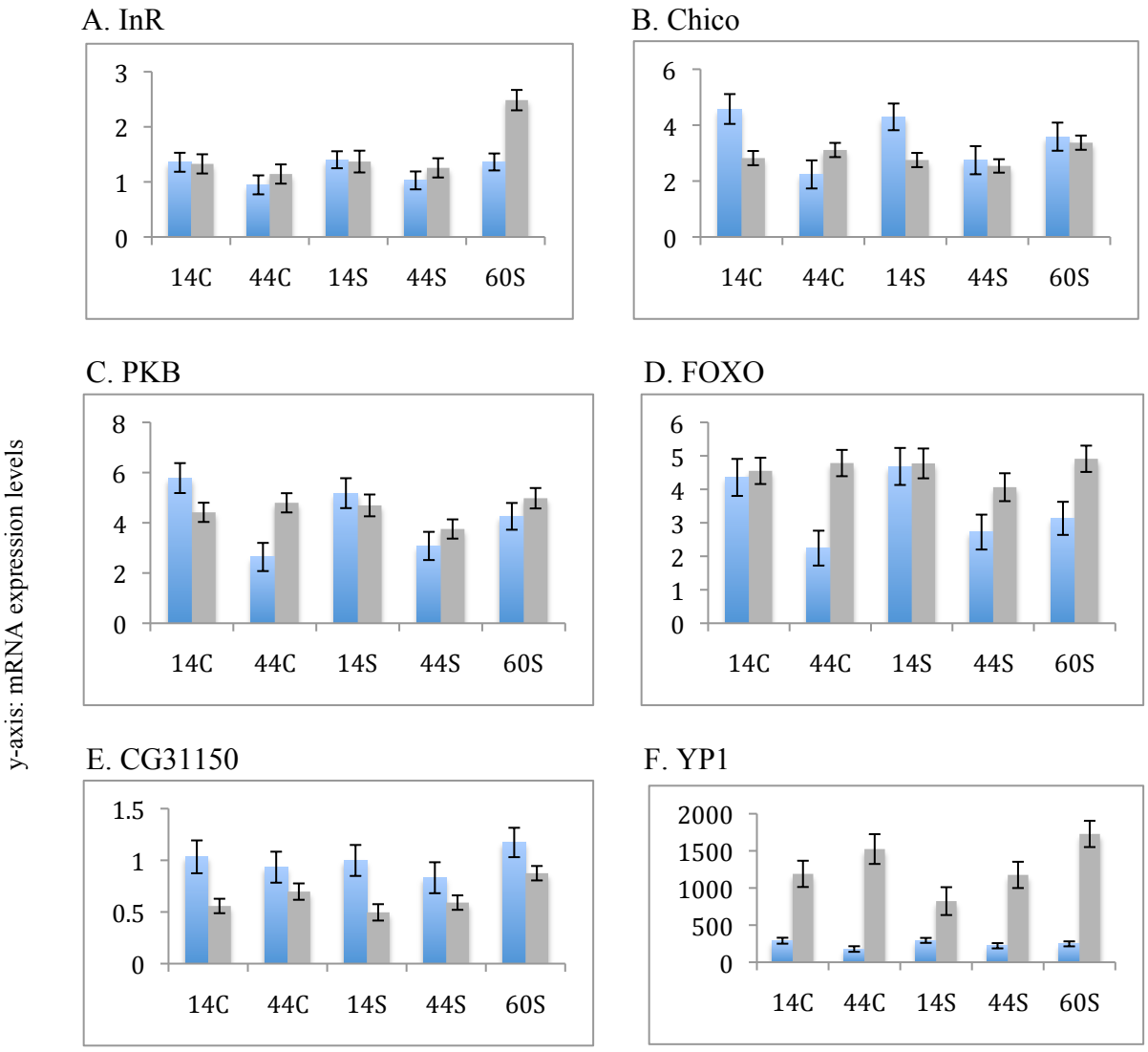


Figure 8. Relative mRNA expression levels in fly abdomen tissue. Letters indicate RNA expression levels for genes in the IIS pathway and vitellogenins (*YP1* and *CG31150*). mRNA levels are shown on the y-axis and Line/Age combinations are shown on the x-axis. Expression bars for set 2 fly lines are coded in blue and set 3 fly lines in grey.



CHAPTER 3

IDENTIFICATION OF MOLECULARLY CHARACTERIZED AND NOVEL BIOCHEMICAL PATHWAYS INVOLVED IN LIFE-HISTORY DIVERGENCE IN FLIES SELECTED ON AGE AT REPRODUCTION

3.1. Introduction

Phenotypic variation in life history strategies within and between species has been extensively documented at the whole-organism level (Stearns, 1992; Roff, 2002). Variation in reproductive strategies arises in part because to maximize fitness, organisms need to perceive and integrate environmental signals (i.e. nutrient availability, temperature, light, presence of pathogens) to correctly allocate limiting resources between life history-traits. Differential allocation of resources leads to negative correlations between these traits. One of the most widely documented tradeoffs occurs between reproduction and lifespan. In insect species, research on energy allocation budgets has shown that resources used in reproduction cannot simultaneously be used in somatic repair and maintenance (Zera and Larsen, 2001; Zhao and Zera, 2002; Min et al., 2006; Judd et al., 2010). Given that life-history traits directly influence fitness of individuals, understanding the molecular mechanisms that regulate their evolutionary history will allow us to link the genetic architecture of these traits to the ecological factors that shape them. This will ultimately help us understand how organisms adapt to their environment (Ferea et al., 1999; Bochdanovits et al, 2003; Townsend et al., 2003; Olesiak et al, 2005; Matzkin et al., 2006; Laayouni et al., 2007; St-Cyr et al., 2008; Burke and Rose, 2009; Stapley et al, 2010; Wang et al, 2011).

Research on the physiological basis of life-history tradeoffs has implicated changes in metabolism and stress resistance as important factors in the determination of reproductive schedules (Service et al., 1985; Service et al., 1987; Rose et al., 1992; Djawdan et al., 1996;

Harshman et al., 1999; Arking et al., 2000; Arking et al., 2002). However, the genetic mechanisms that have been acted upon by natural selection to modify life-history traits remain poorly understood (Stearns and Magwene, 2003; Roff, 2007). Phenotypic variation within and between populations has been associated with variation in genome-wide transcript abundance (Olesiak et al., 2002). Because organisms need to perform coordinated physiological processes to ensure proper functioning, genome-wide scans can allow us to globally investigate how energy allocation patterns lead to tradeoffs between life-history traits at the molecular level (Olesiak et al., 2002; Bochdanovits and de Jong, 2004; Aubin-Horth et al., 2005; Giger et al., 2006; Giger et al., 2008).

To investigate potential molecular mechanisms that accompany the evolution of divergent life-histories, I coupled experimental evolution in *D. melanogaster* with microarray technology. Specifically, I compared changes in gene expression across five ages and two generations in replicated fly lines selected on age at first reproduction relative to their paired control lines. Previous experiments that have tracked changes in gene expression with age in fruit flies have shown that older flies show a decline in expression of genes involved in metabolism of sugars, reproductive system (i.e. oogenesis), energy metabolism (i.e. oxidative phosphorylation, TCA cycle), electron transport chain, and protein synthesis. Genes involved in immune response, heat shock proteins, and antioxidant genes generally increase expression with age (Zou et al., 2000; Landis et al., 2004; Girardot et al., 2006; Pletcher et al., 2002). By limiting energy acquisition through caloric restriction, Pletcher et al (2002) showed that flies under this dietary regime had reduced expression of genes involved in protein metabolism, oogenesis, DNA replication and DNA repair proteins. Caloric restriction also leads to increased oxidative stress resistance (Tettweiler et al., 2005).

Given that organisms experience changes in metabolism, reproductive ability, and tolerance to environmentally imposed stress with age, evolutionary changes in life histories should be associated with changes in energy allocation contingent upon molecular pathways whose end products contribute to reproduction, and somatic maintenance (i.e. energy storage, stress resistance and immunity). I used genome-wide scans to uncover potential molecular pathways involved in the evolution of life-history divergence between selection and control lines. Evaluating genome-wide changes in transcript abundance also allowed me to observe whether genes that have previously been shown to regulate tradeoffs in model organisms (i.e. IIS and TOR pathways) such as roundworms, fruit flies, and mice are involved in life history divergence in flies derived from natural populations. In other words, does the evolution of divergent life-histories always occur through differences in conserved nutrient signal transduction pathways? Alternatively, can we identify novel genes/pathways that contribute to life-history divergence in nature?

3.2. Methods

I used a whole-genome expression assay to identify molecularly characterized and/or novel gene pathways that mediate life history evolution in the paired S-C lines described in chapter 2, section 2.2. Expression changes were monitored across generations of selection, and throughout the life span of the fly, so time-course data in two dimensions were collected. The goal of this experiment was to detect genes and molecular pathways that consistently diverge in expression between lines.

3.2.1. Generation- and age-specific sample collection

Unmated female flies from all three S-C pairs of fly lines were collected at generations 20 and 35 of selection for increased age at reproduction. They were aged in 8-dram vials with cornmeal media for a period of 1, 5, 15, 30, and 50 days of age. During this period, flies were maintained on an incubator at 25C on a 12L:12D light cycle and transferred to fresh media every week. Once they reached the pertinent age, they were lightly anesthetized with CO_2 and frozen in 1.5 ml eppendorf tubes already placed on dry ice. All flies were frozen at 9 a.m. to avoid circadian effects on gene expression. Eppendorf tubes containing frozen flies were stored in the -80C freezer for later dissection. Flies were dissected on dry ice to obtain two tissues: Heads and Abdomens. I generated 240 experimental samples from two generations of selection (20 and 35), three fly sets (1, 2, and 3), two lines (S and C), 2 tissues (head and abdomen), 5 age classes (1, 5, 15, 30, and 50 days), and two biological replicates (1 and 2). Twenty-four samples were missing at time of dissection: twenty from generation 20 set 3C, and four from generation 20 set 1S Day 50.

3.2.2. Generation- and age-specific gene expression

Total RNA was extracted from two independent pools of seven virgin female flies for each population/age/tissue combination using the PicoPure RNA isolation kit (Arcturus). RNA was amplified using Ambion's MessageAmpII aRNA Amplification Kit (Applied Biosystems), and reverse transcribed to cDNA using the SuperScript III protocol (Invitrogen Life Technologies). Quality of total RNA, amplified RNA, and cDNA was assessed using the Experion bioanalyzer (Bio-Rad).

Samples were labeled using Roche-Nimblegen's One Color DNA Labeling Kit and hybridized to one partition of Roche-Nimblegen's *D. melanogaster* 12x135K expression arrays. Each array contains 16,637 target genes with eight probes per gene (DM5.7 genome build).

Arrays were scanned using GenePix 4000B Scanner and Roche NimbleScan software. Four samples were removed from the analysis due to poor labeling, leaving 212 arrays in the analysis. All samples were labeled, hybridized, and scanned at the Roche-Nimblegen approved facility located on the Florida State University campus.

3.2.3 Microarray Data Analysis

Statistical analysis was conducted using SAS software, version 9.2 of the SAS system for Windows (SAS Institute Inc, 2010). Gene expression levels underwent RMA normalization (Irizarry et al., 2003) as implemented in NimbleScan software. Normalized expression levels for each gene were analyzed using a general linear model ANOVA with set, line, age, and generation as fixed effects and replicate arrays as random effects. To correct for multiple testing, I used a $Q < 0.05$ false-discovery rate criterion for significance of any of the terms in the ANOVA model (Benjamini and Hochberg, 1995).

Functional annotation of genes was performed using the batch download function in FlyBase to generate tables with each gene name/symbol, chromosome location, and molecular function of genes differentially expressed at FDR $Q < 0.05$ (Tweedie et al., 2009). Gene ontology (GO) enrichment analyses for the categories of biological process and molecular function, were conducted using DAVID bioinformatics resources (Dennis et al., 2003; Huang et al., 2009).

Changes in transcription levels were calculated as an S/C ratio using normalized log-transformed transcript data, where the least square mean value in C individuals was subtracted from the least square mean value in S individuals for each effect or interaction under study. Hierarchical clustering was conducted using the Euclidean distance method and average-linkage agglomerative algorithm implemented in Genesis software (Sturn et al., 2002). Dendograms

were combined with heat maps to represent color-coded expression intensities of genes. Profile plots depicting changes in expression patterns over time within a cluster were also generated using Genesis software.

3.3 Results

3.3.1 Patterns of differential expression

Four hundred and sixty eight genes showed differential expression between selection and control lines. Of these, 277 were differentially expressed in abdomens and 168 were differentially expressed in heads. The overlap between genes differentially expressed in both tissues was sixteen. A large number of genes showed differential expression with age: 7472 in abdomen tissue and 4076 in head tissue (Table 7).

3.3.2. Enrichment of functional groups associated with life-history divergence

Table 8 lists genes that were differentially expressed between S and C lines (line effect) or that showed differences between lines with age (line*age interaction) in abdomen tissue. Genes are grouped by cluster, and their name, location in the genome, and molecular function are provided. Gene ontology analysis revealed a significant enrichment of the biological process of oxidation-reduction (29 genes) in the list of genes differentially expressed between S and C lines in abdomen tissue (Table 9). This is a metabolic process that results in the removal or addition of electrons to or from a substance. Child terms of this biological process are electron transport chain, lipid oxidation, NADPH oxidation, among others. Although only the molecular function of the genes is provided in Table 8, this table shows that several genes are involved in electron carrier activities, and oxido-reductase activities (i.e. *Cyt-b*, *coI*, *coIII*, *ND4*, *ND5*). Cytochrome c oxidases are part of the electron transport chain in mitochondria and are hypothesized to regulate

the rate of electron flow through the electron transport chain. This suggests that there could be a difference between S and C lines in efficiency of energy production.

In the molecular function category, genes differentially expressed between S and C lines in abdomen tissue were enriched for the categories of oxygen transporter and nutrient reservoir activity (Table 9). The latter category is of particular interest because it implies that S and C lines are using resources in a different way. This suggests that S lines store nutrients when they are not investing them in reproduction.

The list of genes differentially expressed between S and C lines across age classes and generations (line*age*generation interaction) in abdomen tissue, was significantly enriched for the molecular function of Glucuronosyltransferase activity (Table 9). Proteins with this molecular function perform detoxification functions and have been implicated in DDT resistance in fruit flies.

In head tissue, after correction for multiple comparisons there were no significantly enriched gene ontology terms for genes differentially expressed between S and C lines (line effect), genes showing differential expression between lines with age (line*age interaction), or genes showing differential expression between lines across age and generation (line*age*generation interaction).

3.3.3 Clustering Analysis

A primary goal of this study was to identify genes that differ in expression between fly lines with divergent life histories and to understand their implication in the regulation of tradeoffs between reproduction and lifespan. Hierarchical clustering allowed me to group together genes that showed similar age-specific expression profiles for abdomen and head tissue (Figures 9A, 9B). On these heat maps, ratios of transcript levels between selection and control

lines are color coded in red and blue. Red represents an increase in transcript level (S higher than C) and blue represents a decrease (S lower than C).

To investigate how trends in differential expression between S and C lines relate to phenotypic differences in lifespan and reproduction, I plotted changes in expression over time for groups of genes identified by the hierarchical clustering analysis. This analysis identified genes with similar patterns of gene expression between lines and ages. For example, cluster 5 depicts changes in expression of seven genes that exhibit similar behavior: higher expression in S vs. C lines from day 1 until day 30 (Figure 10A). Six of these genes (*Fbp1*, *Fbp2*, *Lsp1alpha*, *Lsp1beta*, *Lsp1gamma*, *Lsp2*) are involved in nutrient reservoir activity.

Cluster 8 shows genes with small differences in expression between S and C lines from days 1 through 15, increased expression in S relative to C lines at day 30 and a decrease in expression at day 50 (Figure 10A). Some of the genes that belong to this cluster include genes involved in oogenesis and germ cell development (*mdy*, *bsk*, *CG7194, 14-3-3zeta*, *Tm1).* Four genes that previously have been implicated in regulation of lifespan and reproduction in model organisms were found in this cluster. They are *Pten*, *il-6*, *sug*, and *JNK*. The first three are involved in insulin signaling, whereas JNK interacts with insulin signaling and is involved in immune response in *Drosophila*.

Patterns of gene expression for genes involved in stress resistance and immune response activities varied. For example, *CG4009* is involved in response to oxidative stress and showed higher expression in S vs. C lines with a sharp increase in expression at day 30 (Figure 10A, Cluster 1). *TotA* is involved in response to stress (heat, cold, bacteria, UV, oxidative stress, and desiccation), and shows high expression in S vs. C lines throughout life (Figure 10A, Cluster 6). *CG10211* and *CG5873* both involved in oxidative stress response showed higher expression in S

vs. C lines early in life but no differential expression at later age classes (Figure 10A, Cluster 7). Four genes involved in immune response (*JNK*, *GNBP1*, *CG3829*, *PARP*) show higher expression in S lines later in life (Figure 10A, Cluster 8). In head tissue, three genes involved in immune response (*CG3088*, *CG8329*, *CG9676*) show increased expression with age in S vs. C lines (Figure 10B, Cluster 1).

Clustering analysis of genes with significant line or line*age interaction effects in head tissue, showed five groups of genes with similar expression. Profile expression plots for these genes are shown in figure 10B. Cluster 2 shows genes that are expressed more highly in S than in C lines particularly earlier in life. These include G-protein coupled receptors involved in sensory perception of light (*Rh2*, *Rh3*, *endoA*), and odors (*or49b*). This cluster also contains serotonin receptors *5HT2* and *5HT1B* and the Fork head transcription factor *FoxP*. Cluster 4 contains many genes involved in translation such as *mRpL17*, *mRpL39*, *mRpS34*, *mRpL24*, *RpL7*, *RpL24*, *mRpS10*, translation initiation such as *CG31957*, and translation elongation such as *eEF1delta* (Figure 10B). Genes in this cluster show overall low expression throughout lifespan in S relative to C lines suggesting a role for differential translation in the heads of S lines vs. C lines.

Analysis of genes with significant line*age*generation interaction effects was expected to reveal signatures of selection across generations. My expectation was that these genes would show: patterns of higher constitutive expression, faster or greater expression, or delayed expression across ages. There were 304 genes that were significantly differentially expressed between lines, across age and generations for abdomen tissue, and only 26 genes for head tissue (Appendix A, B, C). Patterns of expression across ages and generations were less interpretable than those for line and line*age effects (Appendix D).

3.4. Discussion

Differential energy allocation patterns between physiological processes that regulate lifespan and reproduction are considered a primary cause of the widely documented tradeoff observed between these two life-history traits. Using genome-wide scans to compare changes in gene expression between flies with divergent life-histories, I successfully identified 468 candidate genes associated with the regulation of tradeoffs between lifespan and reproduction. This number is comparable to the one found by Sarup et al. (2010) in an experiment of selection for increased lifespan in male flies. My results identified potential differences in energy metabolism and storage as main contributors to life-history divergence. This is consistent with the idea that energy allocation patterns influence the evolution of life-history traits.

Genes identified in the significantly enriched biological process of oxidation-reduction in abdomen tissue, are involved in ATP production, electron transfer in the respiratory chain, electron carrier activities, ubiquinone biosynthesis and NADP-binding activities. Mutations in genes involved in the electron transport chain (i.e. cytochrome oxidase genes) have been shown to reduce lifespan in fruit flies (Liu et al., 2007). Additionally, mutations in *cox* genes in fruit fly cells lead to reduced ATP production (Mandal et al., 2005). Genes involved in the transport of oxygen were also significantly enriched in abdomen tissue. For example, *globin-1* is expressed in tracheal cells and in the fat body. This gene is thought to control oxygen flow from the tracheal system to cells. Taken together, these results suggest that there are differences in energy production between S and C lines. These differences seem to be influenced by electron flow in mitochondria and oxygen transport to cells.

Energy storage also contributes to differences between S and C lines. This is evidenced by the enrichment of genes involved in nutrient reservoir activity. Genes in this category are involved in amino acid storage. These genes are up-regulated earlier in life in S lines, whereas genes that participate in energy metabolism and reproduction are up-regulated later in life in S lines. This pattern suggests that when reproduction occurs S lines increase energy production geared towards this energetically costly process. This pattern could also be consistent with a signature of delayed aging in S lines.

Organisms need to detect and translate environmental signals to coordinate metabolism and growth. Sensory perception has been implicated in the regulation of lifespan and reproduction in *C. elegans* and *D. melanogaster* (Apfeld and Kenyon, 1998; Libert et al., 2007). Although there was no enrichment of differentially expressed genes involved in sensory perception in my study, I did observe genes involved in light and odor perception that were differentially expressed in head tissue of S and C lines. In an experiment by Sarup et al. (2010) where flies were selected for increased lifespan, there was an enrichment of genes involved in light detection. Moreover, a recent experiment using p-element insertions to identify genes that influence lifespan found an over-representation of genes affecting detection of light and abiotic stimuli in female flies (Magwire et al., 2010).

Immunity and oxidative stress resistance are physiological processes that influence lifespan and reproduction (Landis et al., 2004; Libert et al., 2006; DiAngelo et al., 2009). In the current study, I found genes involved in oxidative stress resistance and in the immune response to be differentially expressed between S and C lines. Because these genes have different expression profiles (i.e. constitutive up-regulation, higher expression earlier in life, or higher

expression late in life), at this time it is not possible to conclude how they contribute to observed differences in lifespan and reproduction between my fly lines.

Another goal of this study was to examine differences in gene expression that occur in S vs. C lines across ages and generations. This would allow me to see how the behavior of genes that differ between lines changed across generations and examine the contribution of these genes to life-history divergence. My expectation was to observe some genes in which differences in expression at generation 20 became more exaggerated at generation 35. However, changes in gene expression patterns across generations were generally different from this expected pattern, and sometimes showed opposite trends across generations. This pattern is more consistent with genetic drift than directional selection. Given that I only studied changes in expression across only two generations of selection on age at reproduction at the present time I cannot conclude if genes that are significantly different for the line*age*gen interaction are the ones that cause divergence in life histories. To answer this question it would be helpful to screen the genome for changes in gene expression at the starting and earlier generations of selection. Additionally, a sequencing approach would allow me to track changes at the DNA sequence level to see how these have influenced the evolution of life-history divergence throughout different generations.

QTL mapping studies in flies derived from natural populations have been employed to investigate the molecular underpinnings of variation in life-histories (Nuzdhin et al., 1997; DeLuca et al., 2003; Geiger-Thornsberry et al., 2004; Carbone et al., 2006). These studies have reported that novel candidate genes and previously characterized genes such as *FOXO* influence lifespan (Magwire et al., 2010). Sarup et al. (2010) reached a similar conclusion based on their experiment using male flies selected for increased lifespan. My findings are in agreement with those from QTL studies and Sarup et al. (2010). I was able to identify both novel and previously

characterized genes (i.e. *JNK, Pten*) that contribute to the evolution of divergent life-histories in female fruit flies. These results suggest that the evolution of divergent-life histories occurs through changes in conserved signal transduction pathways but also through changes in other pathways. It also provides support for the use of both candidate gene and unbiased genome-wide scan approaches to fully understand the genetic mechanisms of life-history tradeoffs.

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Table 7. Number of differentially expressed genes for Abdomen and Head tissue at FDR <.05

Effect	Abdomen	Head
Age	7472	4076
Line	228	166
Generation	3072	704
Age*Line	72	20
Age*Generation	4517	579
Line*Generation	22	25
Age*Line*Generation	304	26

Table 8. Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 1	yellow-g2	3L	62D5-62D5	None available
Cluster 1	CG4009	3R	89E10-89E10	peroxidase activity
Cluster 2	CG5756	2R	55B5-55B5	chitin binding
Cluster 2	HP1b	X	8C4-8C4	chromatin binding
Cluster 2	His4:CG33891	2L	39D5-39D5	DNA binding
Cluster 2	geminin	2R	42C2-42C2	DNA binding
Cluster 2	RfC3	2L	31E1-31E1	DNA binding; ATP binding
Cluster 2	CG4594	2L	30E4-30F1	dodecenoyl-CoA delta-isomerase activity
Cluster 2	ferrochelatase	3R	100D2-100D2	ferrochelatase activity
Cluster 2	CG33635	2L	21B3-21B3	identical protein binding
Cluster 2	Ilp6	X	3A1-3A1	insulin receptor binding
Cluster 2	CG3570	2R	60D9-60D9	None available
Cluster 2	CG11137	3L	80B1-80B1	None available
Cluster 2	msd1	3L	61F6-61F6	None available
Cluster 2	CG14985	3L	64A3-64A3	None available
Cluster 2	msd5	3L	61F6-61F6	None available
Cluster 2	CG5194	3L	66F1-66F1	None available
Cluster 2	CG11999	3R	82F10-82F10	None available
Cluster 2	CG34035	3R	90A6-90A6	None available
Cluster 2	CG5359	3R	85F5-85F5	None available
Cluster 2	CG42557	3R	99C7-99C7	None available
Cluster 2	CG10038	3R	87B7-87B7	None available
Cluster 2	CG41457-RA	Unknown	Unknown	None available
Cluster 2	CG12056	Unknown	Unknown	None available
Cluster 2	Putative proteasome inhibitor	Unknown	Unknown	None available
Cluster 2	CG11164	X	12B2-12B2	None available
Cluster 2	CG13373	X	1B4-1B4	None available
Cluster 2	CG1678	X	20A1-20A1	None available
Cluster 2	Kmn1	X	10C6-10C7	None available

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	CG10907	3L	68D6-68D6	peptidyl-prolyl cis-trans isomerase activity
Cluster 2	CG6182	3R	95E6-95E6	Rab GTPase activator activity
Cluster 2	dbe	2L	21E2-21E2	RNA binding
Cluster 2	MED8	2R	56F16-56F16	RNA polymerase II transcription mediator activity
Cluster 2	CG16997	2L	33C4-33C4	serine-type endopeptidase activity
Cluster 2	Fad2	3L	68A1-68A1	stearoyl-CoA 9-desaturase activity
Cluster 2	eIF2B-beta	X	3B2-3B2	translation initiation factor activity
Cluster 2	CG13163	2R	48F7-48F8	translational initiation factor activity
Cluster 2	CG17359	3L	70C11-70C11	zinc ion binding
Cluster 2	CG31457	3R	94E1-94E1	zinc ion binding
Cluster 2	CG8319	3R	85E1-85E1	zinc ion binding
Cluster 3	CG10877	3R	92F1-92F2	catalytic activity
Cluster 3	CG10694	3R	95E1-95E1	damaged DNA binding
Cluster 3	CG30401	2R	58B1-58B1	DNA binding
Cluster 3	Cyp318a1	X	11A2-11A2	electron carrier activity
Cluster 3	O-fut2	X	2A4-2A4	fucosyltransferase activity
Cluster 3	CG33156	2R	50B1-50B1	NAD+ kinase activity
Cluster 3	CG13947	2L	21E2-21E2	None available
Cluster 3	CG14346	2L	22A1-22A1	None available
Cluster 3	CG14346	2L	22A1-22A1	None available
Cluster 3	CG15824	2L	21E2-21E2	None available
Cluster 3	CG3104	2L	23B5-23B5	None available
Cluster 3	CG33003	2L	24F1-24F1	None available
Cluster 3	CG4785	2L	21F1-21F1	None available
Cluster 3	slmo	2L	26B3-26B3	None available
Cluster 3	Fsn	2R	49F10-49F10	None available
Cluster 3	CG13923	3L	62B4-62B4	None available
Cluster 3	dro4	3L	63D1-63D1	None available
Cluster 3	CG11550	3R	100D1-100D1	None available

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 3	mey	3R	100C4-100C4	None available
Cluster 3	CG4433	3R	92C1-92C1	None available
Cluster 3	CG4951	3R	98B6-98B6	None available
Cluster 3	CG13562	Unknown	Unknown	None available
Cluster 3	CG2750	Unknown	Unknown	None available
Cluster 3	Nitric oxide synthase	Unknown	Unknown	None available
Cluster 3	Putative ubiquinone biosynthesis	Unknown	Unknown	None available
Cluster 3	RNA 3'-terminal phosphate cyclase	Unknown	Unknown	None available
Cluster 3	CG12057	X	8C17-8C17	None available
Cluster 3	meso18E	X	18E2-18E3	None available
Cluster 3	CG32537	X	18B7-18B8	None available
Cluster 3	tty	X	19F4-19F5	None available
Cluster 3	interacting protein	Unknown	Unknown	None available
Cluster 3	monooxygenase COQ6	Unknown	Unknown	None available
Cluster 3	CG10222	3L	70A8-70A8	nucleotide binding
Cluster 3	Ppox	3R	96A14-96A14	oxygen-dependent protoporphyrinogen oxidase activity
Cluster 3	rpr	3L	75C6-75C6	phospholipid binding
Cluster 3	CG32770	X	4D5-4D5	potassium channel activity
Cluster 3	CG15072	2R	55E8-55E9	protein serine/threonine kinase activity
Cluster 3	CG31832	2L	35B8-35B8	receptor binding
Cluster 3	Crg-1	X	3F1-3F2	RNA polymerase II transcription factor activity
Cluster 3	fry	3L	67C3-67C4	transcription activator activity
Cluster 3	CG30156	2R	42E1-42E1	unfolded protein binding
Cluster 3	CG12299	2L	32A5-32A5	zinc ion binding
Cluster 3	CG8786	3L	76B11-76B11	zinc ion binding
Cluster 3	Zpr1	X	8C14-8C14	zinc ion binding
Cluster 4	CG34040	2R	58A2-58A2	None available
Cluster 4	CG15025	3R	92A6-92A6	None available
Cluster 4	CG33346	3R	98E1-98E1	None available

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 4	CG17751	3R	92A10-92A10	secondary active organic cation transmembrane transporter activity
Cluster 5	CG11538-RA	Unknown	Unknown	None available
Cluster 5	Fbp2	2L	30B3-30B3	nutrient reservoir activity
Cluster 5	Lsp1alpha	X	11A12-11A12	nutrient reservoir activity
Cluster 5	Lsp1beta	2L	21E2-21E2	nutrient reservoir activity
Cluster 5	Lsp1gamma	3L	61A6-61A6	nutrient reservoir activity
Cluster 5	Lsp2	3L	68F5-68F5	nutrient reservoir activity
Cluster 5	Fbp1	3L	70D2-70D2	protein transporter activity; oxygen transporter activity
Cluster 6	CG11034	2L	25F5-25F5	dipeptidyl-peptidase activity
Cluster 6	TotA	3R	93A2-93A2	None available
Cluster 6	CG31668	2L	22D1-22D4	transporter activity
Cluster 7	CG9541	2L	29F6-29F6	adenylate kinase activity
Cluster 7	Ama	3R	84A5-84A5	antigen binding
Cluster 7	Dscam	2R	43A4-43B1	axon guidance receptor activity; protein homodimerization activity
Cluster 7	e	3R	93C7-93D1	beta-alanyl-dopamine synthase activity
Cluster 7	Ect3	3R	87A7-87A7	beta-galactosidase activity
Cluster 7	CG1090	3R	82A4-82A4	calcium, potassium:sodium antiporter activity
Cluster 7	Gasp	3R	83D4-83D4	chitin binding; structural constituent of peritrophic membrane
Cluster 7	CG1869	3L	63B1-63B1	chitinase activity
Cluster 7	CG9519	X	12F6-12F6	choline dehydrogenase activity
Cluster 7	tw	X	1C4-1D4	dolichyl-phosphate-mannose-protein mannosyltransferase activity
Cluster 7	Cyp316a1	3L	66A2-66A2	electron carrier activity
Cluster 7	GATAe	3R	89A12-89A12	general RNA polymerase II transcription factor activity
Cluster 7	CG3961	3L	75E3-75E4	long-chain fatty acid-CoA ligase activity
Cluster 7	verm	3L	76C2-76C3	low-density lipoprotein receptor activity
Cluster 7	CG4500	2L	34F1-34F1	None available
Cluster 7	nimC2	2L	34E5-34E5	None available
Cluster 7	CG34199	2R	56F16-56F16	None available
Cluster 7	CG12488	Unknown	Unknown	None available

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 7	Cda4	X	20C3-20D1	None available
Cluster 7	CG33082	X	1E1-1E1	None available
Cluster 7	CG8100	3L	70D2-70D2	oxygen transporter activity
Cluster 7	CG10211	2L	36F6-36F6	peroxidase activity
Cluster 7	CG5873	3R	90A6-90B1	peroxidase activity
Cluster 7	comm	3L	71F2-71F2	protein binding
Cluster 7	CG4650	2L	35B5-35B5	serine-type endopeptidase activity
Cluster 7	CG10764	2R	54B16-54B16	serine-type endopeptidase activity
Cluster 7	CG30286	2R	57E9-57E10	serine-type endopeptidase activity
Cluster 7	Cpr49Ac	2R	49A2-49A2	structural constituent of chitin-based larval cuticle
Cluster 7	Gr8a	X	8D2-8D2	taste receptor activity
Cluster 8	alpha-catenin-related	2R	60A14-60A14	actin binding
Cluster 8	Hip1	3L	69E2-69E2	actin binding
Cluster 8	Tm1	3R	88E12-88E13	actin binding
Cluster 8	wupA	X	16F7-16F7	actin binding
Cluster 8	CG2767	3R	84E8-84E8	alcohol dehydrogenase (NADP+) activity
Cluster 8	CG10863	3L	64A1-64A1	aldehyde reductase activity
Cluster 8	CG5361	3R	85F9-85F9	alkaline phosphatase activity
Cluster 8	Mal-B1	2L	33A3-33A4	alpha-glucosidase activity
Cluster 8	CG5322	2L	31E4-31E4	alpha-mannosidase activity
Cluster 8	CG6465	3R	86C4-86C4	aminoacylase activity
Cluster 8	CG6071	3L	68D1-68D1	aminopeptidase activity
Cluster 8	CG31445	3R	99A1-99A4	aminopeptidase activity
Cluster 8	CG18473	3R	85D11-85D12	aryldialkylphosphatase activity
Cluster 8	CG15547	3R	100A6-100A6	ATP binding
Cluster 8	CG4511	3R	86C7-86C7	ATP binding
Cluster 8	ATPCL	2R	52D9-52D11	ATP citrate synthase activity
Cluster 8	CG3164	2L	21B2-21B2	ATPase activity
Cluster 8	CG4822	2L	21B2-21B2	ATPase activity

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 8	CG5853	2L	30F1-30F1	ATPase activity
Cluster 8	cals	4	102F8-102F8	calcium ion binding
Cluster 8	CG31999	4	102A8-102B1	calcium ion binding
Cluster 8	Glt	2L	29E3-29E3	calcium ion binding
Cluster 8	PMCA	4	102B5-102B5	calcium-transporting ATPase activity
Cluster 8	CG32698	X	9A2-9A2	carbonate dehydratase activity
Cluster 8	alpha-Est7	3R	84D9-84D9	carboxylesterase activity
Cluster 8	CG9509	X	13A1-13A1	choline dehydrogenase activity
Cluster 8	mt:CoI	mt		cytochrome-c oxidase activity
Cluster 8	mt:CoIII	mt		cytochrome-c oxidase activity
Cluster 8	mdy	2L	36B1-36B2	diacylglycerol O-acyltransferase activity
Cluster 8	Cyp4ac1	2L	25D2-25D2	electron carrier activity
Cluster 8	Cyp12c1	3L	75D6-75D6	electron carrier activity
Cluster 8	Cyp12e1	3R	86B4-86B4	electron carrier activity
Cluster 8	Cyp6d4	3R	94C1-94C1	electron carrier activity
Cluster 8	CG9649	3R	88A12-88A12	endopeptidase activity
Cluster 8	Pect	2L	34A9-34A9	ethanolamine-phosphate cytidylyltransferase activity
Cluster 8	NtR	2R	58B10-58C1	excitatory extracellular ligand-gated ion channel activity
Cluster 8	Fer1HCH	3R	99F2-99F2	ferrous iron binding
Cluster 8	mthl14	3L	61B3-61B3	G-protein coupled receptor activity
Cluster 8	lectin-28C	2L	28D2-28D2	galactose binding
Cluster 8	CG4335	3R	92E7-92E7	gamma-butyrobetaine dioxygenase activity
Cluster 8	CG32196	3L	75C7-75C7	gamma-glutamylcyclotransferase activity
Cluster 8	CG4302	2R	57D1-57D2	glucuronosyltransferase activity
Cluster 8	Ugt86Dd	3R	86D4-86D4	glucuronosyltransferase activity
Cluster 8	Ugt35a	3R	86D5-86D5	glucuronosyltransferase activity
Cluster 8	CG5976	3L	77C4-77C4	glutaminyl-peptide cyclotransferase activity
Cluster 8	CG6776	3L	66D5-66D5	glutathione transferase activity
Cluster 8	G-salpha60A	2R	60A12-60A13	GTPase activity

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 8	Rab7	3R	95D5-95D5	GTPase activity
Cluster 8	Rab39	X	7B7-7B7	GTPase activity
Cluster 8	Cda4	X	20C3-20D1	hydrolase activity
Cluster 8	bsk	2L	31B1-31B1	JUN kinase activity
Cluster 8	LBR	2R	57F10-57F11	lamin binding
Cluster 8	Mlc1	3R	98A14-98A15	microfilament motor activity
Cluster 8	CG17838	3R	92F12-93A1	mRNA binding
Cluster 8	Parp	3R	81F-81F	NAD+ ADP-ribosyltransferase activity
Cluster 8	mt:ND4	mt		NADH dehydrogenase (ubiquinone) activity
Cluster 8	mt:ND5	mt		NADH dehydrogenase (ubiquinone) activity
Cluster 8	CCHa2	3R	87E8-87E8	neuropeptide hormone activity
Cluster 8	CCHa2	3R	87E8-87E8	neuropeptide hormone activity
Cluster 8	CG13794	2L	28C2-28C2	neurotransmitter transporter activity
Cluster 8	CG13795	2L	28C2-28C2	neurotransmitter transporter activity
Cluster 8	cpx	3R	82A1-82A3	neurotransmitter transporter activity
Cluster 8	Pten	2L	31B1-31B1	non-membrane spanning protein tyrosine phosphatase activity
Cluster 8	Pten	2L	31B1-31B1	non-membrane spanning protein tyrosine phosphatase activity
Cluster 8	CG1674	4	102B1-102B1	None available
Cluster 8	CG10283	2L	36F5-36F5	None available
Cluster 8	CG11592	2L	21B7-21B7	None available
Cluster 8	CG18095	2L	34F1-34F2	None available
Cluster 8	CG31769	2L	34F4-34F5	None available
Cluster 8	CG31886	2L	29F5-29F5	None available
Cluster 8	Lamp1	2L	39E2-39E2	None available
Cluster 8	CG8852	2L	23F6-24A1	None available
Cluster 8	CG17665	2R	h44-h46	None available
Cluster 8	CG18609	2R	55E11-55E11	None available
Cluster 8	Vps13	2R	43D7-43E1	None available
Cluster 8	CG3907	2R	60B3-60B4	None available

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 8	l(2)05510	2R	57A5-57A6	None available
Cluster 8	Tsp42Ea	2R	42E4-42E4	None available
Cluster 8	CG13032	3L	73B6-73B6	None available
Cluster 8	CG32195	3L	75B10-75B10	None available
Cluster 8	CG33274-RB	3L	66C3-66C4	None available
Cluster 8	cp309	3L	71B2-71B4	None available
Cluster 8	nvd	3L	Unknown	None available
Cluster 8	CG40351	3L	Unknown	None available
Cluster 8	CG7194	3L	66C5-66C5	None available
Cluster 8	gk	3L	75B11-75B12	None available
Cluster 8	Sug	3L	68D2-68D2	None available
Cluster 8	CG10560	3R	96D1-96D1	None available
Cluster 8	CG14245	3R	97C5-97C5	None available
Cluster 8	CG14280	3R	91F13-91F13	None available
Cluster 8	CG14292	3R	91D4-91D4	None available
Cluster 8	CG33330	3R	88D3-88D3	None available
Cluster 8	c(3)G	3R	89A5-89A5	None available
Cluster 8	CG31106	Unknown	Unknown	None available
Cluster 8	CG31189	Unknown	Unknown	None available
Cluster 8	CG32712-RA	Unknown	Unknown	None available
Cluster 8	CG40769	Unknown	Unknown	None available
Cluster 8	CG41243	Unknown	Unknown	None available
Cluster 8	CG41581	Unknown	Unknown	None available
Cluster 8	Unknown	Unknown	Unknown	None available
Cluster 8	Probable insulin-like peptide 6	Unknown	Unknown	None available
Cluster 8	Protein KRI1 homolog	Unknown	Unknown	None available
Cluster 8	CG12470	X	1A1-1A1	None available
Cluster 8	CG1572	X	10C5-10C5	None available
Cluster 8	CG34325	X	15A3-15A3	None available

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 8	Or67a	3L	67B11-67B11	odorant binding
Cluster 8	Or1a	X	1A2-1A2	odorant binding
Cluster 8	CG3301	3R	93D2-93D2	oxidoreductase activity
Cluster 8	CG9360	X	10E2-10E2	oxidoreductase activity
Cluster 8	glob1	3R	89A8-89A8	oxygen transporter activity
Cluster 8	GNBP1	3L	75D6-75D6	peptidoglycan binding
Cluster 8	Atf6	2R	41D4-41E1	protein homodimerization activity
Cluster 8	Lk6	3R	86E18-86E18	protein kinase activity
Cluster 8	CaMKI	4	102C1-102C1	protein serine/threonine kinase activity
Cluster 8	Dyrk3	4	102F8-102F8	protein serine/threonine kinase activity
Cluster 8	CG17698	3L	80F9-80F9	protein serine/threonine kinase activity
Cluster 8	CG7497-RA	3L	74E3-74E4	protein serine/threonine kinase activity
Cluster 8	Sema-5c	3L	68F2-68F2	receptor activity
Cluster 8	CG7668	3L	76E1-76E1	receptor binding
Cluster 8	CG15611	2R	53F10-53F11	Rho guanyl-nucleotide exchange factor activity
Cluster 8	Tif-IA	2L	40F6-40F7	RNA polymerase I transcription factor activity
Cluster 8	CG4061	X	2C10-2C10	RNA-3'-phosphate cyclase activity
Cluster 8	CHKov1	3R	96D1-96D1	RNA-directed DNA polymerase activity
Cluster 8	CG6385	2R	54E8-54E8	sarcosine dehydrogenase activity
Cluster 8	CG3829	2R	60E11-60E11	scavenger receptor activity
Cluster 8	crc	2L	39C2-39C3	sequence-specific DNA binding transcription factor activity
Cluster 8	CYLD	2L	31C7-31D1	sequence-specific DNA binding transcription factor activity
Cluster 8	CG5390	2L	31D1-31D1	serine-type endopeptidase activity
Cluster 8	CG30289	2R	57E9-57E9	serine-type endopeptidase activity
Cluster 8	Tequila	3L	66F4-66F5	serine-type endopeptidase activity
Cluster 8	CG34454	3L	61B2-61B2	serine-type endopeptidase inhibitor activity
Cluster 8	CG42235	3R	96F8-96F8	sodium-dependent multivitamin transmembrane transporter activity
Cluster 8	Act42A	2R	42A7-42A7	structural constituent of cytoskeleton
Cluster 8	Act57B	2R	57B5-57B5	structural constituent of cytoskeleton

Table 8 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 8	Act87E	3R	87E11-87E11	structural constituent of cytoskeleton
Cluster 8	CG34417	X	6B3-6C1	structural constituent of cytoskeleton
Cluster 8	mRpS5	3L	Unknown	structural constituent of ribosome
Cluster 8	mRpS5	3L	Unknown	structural constituent of ribosome
Cluster 8	RpL7A	X	6B1-6B1	structural constituent of ribosome
Cluster 8	e(r)	X	8B7-8B7	transcription regulator activity
Cluster 8	Eph	4	102D1-102D1	transmembrane receptor protein tyrosine kinase activity
Cluster 8	CG3285	2L	23E4-23E4	transmembrane transporter activity
Cluster 8	CG5973	2L	27F4-27F4	transporter activity
Cluster 8	CG30344	2R	45A12-45A13	transporter activity
Cluster 8	CG31272	3R	86C5-86C5	transporter activity
Cluster 8	CG3091	X	2F2-2F2	transporter activity
Cluster 8	CG6277	3R	97D14-97D14	triglyceride lipase activity
Cluster 8	14-3-3zeta	2R	46E6-46E8	tryptophan hydroxylase activator activity
Cluster 8	mt:Cyt-b	mt		ubiquinol-cytochrome-c reductase activity
Cluster 8	UGP	3L	67B1-67B2	UTP:glucose-1-phosphate uridylyltransferase activity
Cluster 8	CG6045	3R	88F7-88F7	xanthine dehydrogenase activity
Cluster 8	CG4080	3L	67B3-67B4	zinc ion binding
Cluster 8	Unc-115b	3R	85E4-85E4	zinc ion binding
Cluster 8	CG32581	X	13F1-13F1	zinc ion binding
Cluster 8	CG8974	X	13E18-13F1	zinc ion binding

Table 9. Biological processes and molecular functions enriched in abdomen tissue for the combined effects of Line/Line by age, and Line by Age by Generation. Only significantly enriched categories after correction for multiple testing are included.

Effect	Biological process	Count	PValue	Fold Enrichment	FDR
Line and Line*Age	Oxidation reduction	29	1.46E-05	2.43	0.02
	Molecular function				
Line and Line*Age	Oxygen transporter activity	7	1.73E-07	23.95	2.35E-04
	Nutrient reservoir activity	5	3.50E-06	37.07	0.005
Line*Age*Generation	Glucuronosyltransferase activity	8	0.00001	10.65	0.01

Table 10. Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Head tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 1	CG8343	2R	42A13-42A13	mannose binding
Cluster 1	CG3088	3L	67C2-67C2	serine-type endopeptidase activity
Cluster 1	CG8329	3L	67C3-67C3	serine-type endopeptidase activity
Cluster 1	CG9676	X	15A1-15A1	serine-type endopeptidase activity
Cluster 2	CG6071	3L	68D1-68D1	aminopeptidase activity
Cluster 2	CG4511	3R	86C7-86C7	ATP binding
Cluster 2	Atet	2L	24E1-24E1	ATPase activity, coupled to transmembrane movement of substances
Cluster 2	CG14709	3R	86E11-86E11	ATPase activity, coupled to transmembrane movement of substances
Cluster 2	CG2256	X	7D5-7D6	calcium ion binding
Cluster 2	Est-P	3L	69A1-69A1	carboxylesterase activity
Cluster 2	CG5316	3R	92A3-92A3	damaged DNA binding
Cluster 2	CG34365	2R	52A3-52A4	diacylglycerol binding
Cluster 2	Cyp316a1	3L	66A2-66A2	electron carrier activity
Cluster 2	5-HT1B	2R	56B1-56B1	G-protein coupled amine receptor activity; serotonin receptor activity
Cluster 2	Rh2	3R	91D4-91D4	G-protein coupled photoreceptor activity
Cluster 2	Rh3	3R	92C5-92C5	G-protein coupled photoreceptor activity
Cluster 2	pollux	3R	83B9-83C1	integrin binding
Cluster 2	endoA	3R	91D4-91D4	lysophosphatidic acid acyltransferase activity
Cluster 2	CG31427	3R	99A1-99A1	metallopeptidase activity
Cluster 2	CG31427	3R	99A1-99A1	metallopeptidase activity
Cluster 2	Rb97D	3R	97D3-97D3	mRNA binding
Cluster 2	DmsR-1	3L	62D6-62D6	myosuppressin receptor activity; neuropeptide receptor activity
Cluster 2	CG7408	3L	75A7-75A8	N-acetylgalactosamine-4-sulfatase activity
Cluster 2	tutl	2L	24E1-24E1	None available
Cluster 2	CG3588	X	3C3-3C3	None available
Cluster 2	CG15270	2L	35C1-35C2	None available
Cluster 2	CG10283	2L	36F5-36F5	None available
Cluster 2	CG8008	2R	45B1-45B1	None available
Cluster 2	CG13870	2R	56F17-56F17	None available

Table 10 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Head tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	CG16742	2R	57A8-57A8	None available
Cluster 2	CG13917	3L	62A7-62A7	None available
Cluster 2	CG14995	3L	64A5-64A6	None available
Cluster 2	CG14995	3L	64A5-64A6	None available
Cluster 2	CG14995	3L	64A5-64A6	None available
Cluster 2	CG14177	3L	67C2-67C2	None available
Cluster 2	CG14450	3L	80A1-80A1	None available
Cluster 2	CG12947	3R	85E8-85E8	None available
Cluster 2	Osi22	3R	87E4-87E4	None available
Cluster 2	CG15025	3R	92A6-92A6	None available
Cluster 2	CG10883		Unknown	None available
Cluster 2	dpr	2R	57B1-57B2	None available
Cluster 2	MESK2	2R	57E8-57E9	None available
Cluster 2	CG33702	3L	67B11-67B11	None available
Cluster 2	CG33995	2L	25C1-25C1	None available
Cluster 2	Ir10a	X	10B2-10B2	None available
Cluster 2	CG40973		Unknown	None available
Cluster 2	Or49b	2R	49D4-49D4	olfactory receptor activity
Cluster 2	CG15684	3R	92E2-92E2	oxysterol binding
Cluster 2	ced-6	2R	45D7-45D8	protein binding
Cluster 2	PKD	3R	91A2-91A2	protein serine/threonine kinase activity
Cluster 2	CG4030	2R	57B19-57B19	Rab GTPase binding
Cluster 2	Sur-8	3R	90A5-90A5	Ras GTPase binding
Cluster 2	apt	2R	59F1-59F4	RNA polymerase II transcription factor activity
Cluster 2	CG11835	2L	21E2-21E2	sequence-specific DNA binding transcription factor activity
Cluster 2	FoxP	3R	85E5-85E5	sequence-specific DNA binding transcription factor activity
Cluster 2	5-HT2	3R	82C3-82D5	serotonin receptor activity
Cluster 2	CG32082	3L	68A8-68A9	signal transducer activity
Cluster 2	cbt	2L	21D1-21D1	transcription activator activity

Table 10 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Head tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	CG11891	3R	96C8-96C8	transferase activity, transferring phosphorus-containing groups
Cluster 2	CG31668	2L	22D1-22D4	transporter activity
Cluster 2	CG4080	3L	67B3-67B4	zinc ion binding
Cluster 3	Fbp2	2L	30B3-30B3	nutrient reservoir activity
Cluster 3	Fbp1	3L	70D2-70D2	protein transporter activity
Cluster 4	CG5567	3L	75A4-75A4	4-nitrophenylphosphatase activity
Cluster 4	rasp	3L	63B8-63B8	acyltransferase activity
Cluster 4	Aprt	3L	62B9-62B9	adenine phosphoribosyltransferase activity
Cluster 4	Ada	3R	85C4-85C4	adenosine deaminase activity
Cluster 4	SamDC	2L	31D9-31D9	adenosylmethionine decarboxylase activity
Cluster 4	CG31343	3R	93F8-93F8	aminopeptidase activity
Cluster 4	CG13692	2L	21C2-21C2	ARF GTPase activator activity
Cluster 4	CG10973	3L	69E1-69E1	binding
Cluster 4	Nrg	X	7F2-7F4	calcium ion binding
Cluster 4	CG14512	3R	98F12-98F12	carbohydrate binding
Cluster 4	CG10877	3R	92F1-92F2	catalytic activity
Cluster 4	CG15220	X	10D1-10D1	DNA binding
Cluster 4	CG6870	2L	36D1-36D1	electron carrier activity
Cluster 4	fbp	2L	38A2-38A3	fructose 1,6-bisphosphate 1-phosphatase activity
Cluster 4	CG1969	3R	99B9-99B9	glucosamine 6-phosphate N-acetyltransferase activity
Cluster 4	CG12056	X	8C17-8C17	heme binding
Cluster 4	CG18643	3R	86E5-86E5	hydrolase activity, acting on ester bonds
Cluster 4	fh	X	8C14-8C14	iron chaperone activity
Cluster 4	Jheh3	2R	55F8-55F8	juvenile hormone epoxide hydrolase activity
Cluster 4	CG8067	2R	50C23-50C23	methyltransferase activity
Cluster 4	CG11342	3L	64B2-64B2	methyltransferase activity
Cluster 4	CG10466	2L	38A3-38A3	mRNA binding
Cluster 4	CG14291	3R	91D4-91D4	N-sulfoglucosamine sulfohydrolase activity
Cluster 4	CG3683	2R	60D13-60D13	NADH dehydrogenase (ubiquinone) activity

Table 10 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Head tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 4	l(3)neo18	3L	68F5-68F5	NADH dehydrogenase (ubiquinone) activity
Cluster 4	CG3714	2L	24D8-24D8	nicotinate phosphoribosyltransferase activity
Cluster 4	ImpL1	3L	70A7-70A7	None available
Cluster 4	CG14270	X	3D4-3D4	None available
Cluster 4	CG14430	X	6E4-6E4	None available
Cluster 4	CG7267	X	8C4-8C4	None available
Cluster 4	CG9689	X	9A3-9A3	None available
Cluster 4	kek5	X	18C3-18C7	None available
Cluster 4	CG14229	X	18E1-18E1	None available
Cluster 4	CG3652	2L	24E5-24E5	None available
Cluster 4	CG7224	2L	28D3-28D3	None available
Cluster 4	CG6583	2L	33D2-33D2	None available
Cluster 4	CG2611	2L	38D2-38D2	None available
Cluster 4	CG15650	2R	57B5-57B5	None available
Cluster 4	CG13562	2R	60A9-60A9	None available
Cluster 4	CG3894	2R	60E1-60E1	None available
Cluster 4	CG14107	3L	70A7-70A7	None available
Cluster 4	CG10171	3L	70A7-70A8	None available
Cluster 4	CG10171	3L	70A7-70A8	None available
Cluster 4	CG5969	3L	77C4-77C4	None available
Cluster 4	CG10584	3L	78A2-78A5	None available
Cluster 4	CG1142	3R	84D8-84D8	None available
Cluster 4	CG9396	3R	85D25-85D25	None available
Cluster 4	CG7713	3R	90C5-90C5	None available
Cluster 4	CG30005	2R	45F6-45F6	None available
Cluster 4	CG31126	3R	96A14-96A14	None available
Cluster 4	CG32442	3L	78D5-78D5	None available
Cluster 4	CG41541		Unknown	None available
Cluster 4	CG41586		Unknown	None available

Table 10 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Head tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 4	rtp	3R	82F6-82F6	None available
Cluster 4	CG42553	3L	61D2-61D2	None available
Cluster 4	CG33228	2R	60D13-60D13	None available
Cluster 4	CG15675-RB		Unknown	None available
Cluster 4	CG5558-RB		Unknown	None available
Cluster 4	CG14464-RA	2R	41C3-41C3	None available
Cluster 4	CG14303	3R	91B7-91B8	nucleic acid binding
Cluster 4	Wwox	2L	28D3-28D3	oxidoreductase activity
Cluster 4	CG31229	3R	91B8-91B8	P-P-bond-hydrolysis-driven protein transmembrane transporter activity
Cluster 4	Tom20	3L	76E1-76E1	P-P-bond-hydrolysis-driven protein transmembrane transporter activity
Cluster 4	mud	X	12E5-12E6	protein binding
Cluster 4	140up	3R	88A9-88A9	protein transporter activity
Cluster 4	spn-B	3R	88B4-88B4	recombinase activity
Cluster 4	CG7342	3R	92A10-92A10	secondary active organic cation transmembrane transporter activity
Cluster 4	CG5377	3R	94A16-94A16	serine hydrolase activity
Cluster 4	CG4914	3L	70E7-70E7	serine-type endopeptidase activity
Cluster 4	CG11637	3L	75E4-75E4	short-branched-chain-acyl-CoA dehydrogenase activity
Cluster 4	Cpr65Au	3L	65A6-65A6	structural constituent of chitin-based cuticle
Cluster 4	mRpL17	3L	61B3-61B3	structural constituent of ribosome
Cluster 4	mRpL39	3L	71B1-71B1	structural constituent of ribosome
Cluster 4	mRpS34	3L	72E1-72E2	structural constituent of ribosome
Cluster 4	mRpS25	X	12F1-12F1	structural constituent of ribosome
Cluster 4	mRpL24	2L	25B4-25B4	structural constituent of ribosome
Cluster 4	RpL7-like	2L	33C1-33C1	structural constituent of ribosome
Cluster 4	RpL24-like	3R	86E5-86E5	structural constituent of ribosome
Cluster 4	mRpS10	3R	88E3-88E3	structural constituent of ribosome
Cluster 4	Vm26Ab	2L	26A3-26A3	structural constituent of vitelline membrane
Cluster 4	eEF1delta	2L	31B1-31B1	translation elongation factor activity
Cluster 4	CG31957	2L	24C8-24C8	translation initiation factor activity

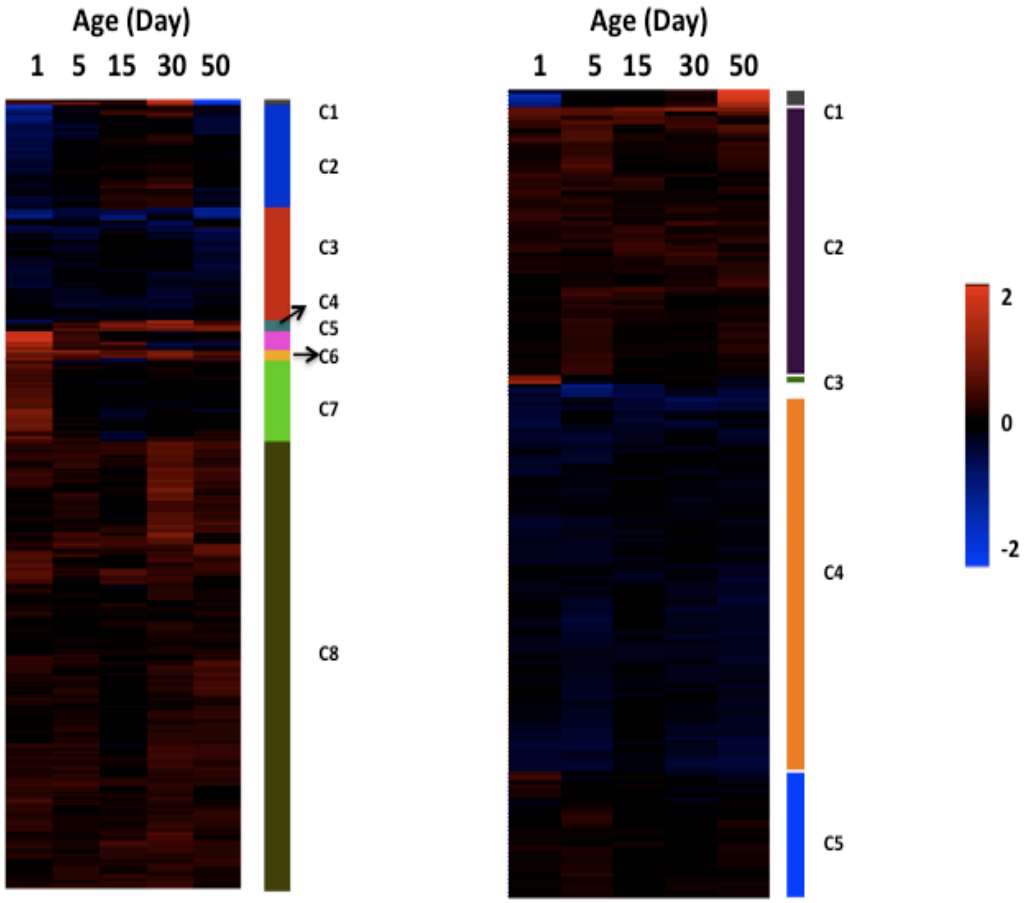
Table 10 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Head tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 4	CG17930	3R	89B12-89B12	transmembrane transporter activity
Cluster 4	CG13993	2L	26B4-26B4	unfolded protein binding
Cluster 5	Chd64	3L	64A6-64A7	actin binding; juvenile hormone response element binding
Cluster 5	CG18809	X	18E5-18E5	cytochrome-c oxidase activity
Cluster 5	Ir21a	2L	21A5-21B1	extracellular-glutamate-gated ion channel activity
Cluster 5	inx7	X	6E4-6E4	gap junction channel activity
Cluster 5	CG17364	3L	70C9-70C9	GTP binding
Cluster 5	NTPase	2L	23C1-23C1	guanosine-diphosphatase activity
Cluster 5	Gyc-89Db	3R	89B18-89B18	guanylate cyclase activity
Cluster 5	Art4	3R	85F4-85F4	histone methyltransferase activity
Cluster 5	Su(z)12	3L	76D4-76D4	histone methyltransferase activity
Cluster 5	Cirl	2R	44D4-44D5	latrotoxin receptor activity; sugar binding
Cluster 5	CG11412	X	1F3-1F4	N-acetyltransferase activity
Cluster 5	CG9866	2L	22E1-22E1	None available
Cluster 5	CG15626	2L	25A8-25A8	None available
Cluster 5	CG8964	2R	48D7-48D7	None available
Cluster 5	CG11242	2R	56D11-56D12	None available
Cluster 5	CG6652	3L	73E4-73E4	None available
Cluster 5	CG14852	3R	88C6-88C6	None available
Cluster 5	CR32027	3L	75E2-75E2	None available
Cluster 5	CG34269	3L	61C3-61C3	None available
Cluster 5	Pmm45A	2R	45A6-45A7	phosphomannomutase activity
Cluster 5	cnk	2R	54B7-54B7	protein binding
Cluster 5	CG12229	3L	74C1-74C1	pyruvate kinase activity
Cluster 5	Sema-5c	3L	68F2-68F2	receptor activity
Cluster 5	HGTX	3L	70E3-70E3	sequence-specific DNA binding transcription factor activity
Cluster 5	ham	2L	37A2-37A4	sequence-specific DNA binding transcription factor activity
Cluster 5	pnr	3R	89A13-89B1	sequence-specific DNA binding transcription factor activity
Cluster 5	Takr86C	3R	86C5-86C5	tachykinin receptor activity

Table 10 (Cont.) Genes with significantly different expression between S and C lines (line effect) and between lines with age (line*age) interaction in Head tissue.

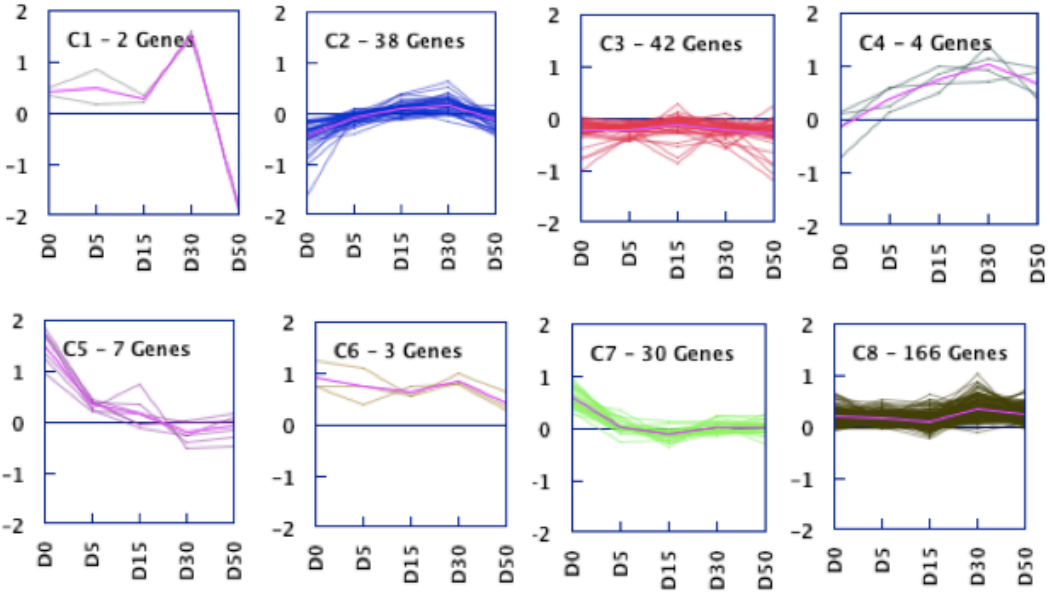
Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 5	CR31054	3R	98B6-98B6	translation elongation factor activity
Cluster 5	ref(2)P	2L	37F1-37F1	zinc ion binding
No Cluster	CG33346	3R	98E1-98E1	hydrolase activity
No Cluster	CG31775	2L	35B5-35B5	None available
No Cluster	CG3770	2R	60E8-60E8	None available

Figure 9. Heat map representation of differentially expressed genes between S and C lines for (A) Abdomen and (B) Head tissue. Each column represents an age-class and each row represents the expression pattern of a gene across all age-classes. The ratios of transcript levels between selection and control lines are color coded in red and blue. Red represents an increase in transcript level and blue represents a decrease. Genes in each tissue/effect combination are grouped in three to eight clusters (C1-C8) obtained by hierarchical clustering analysis based on similarities in expression profiles.

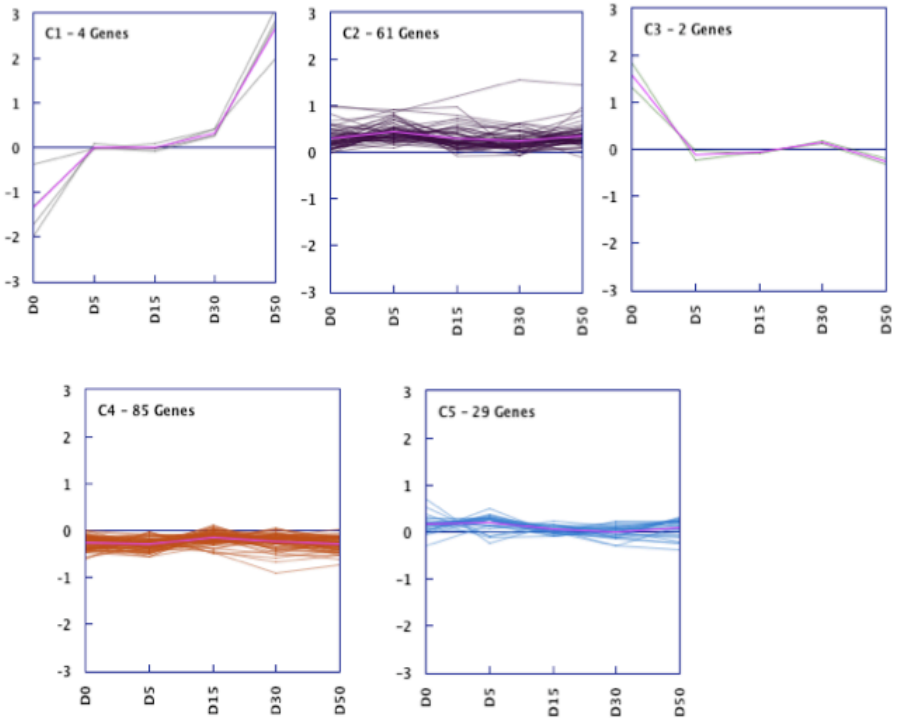


(A) Abdomen (B) Head

Figure 10. Gene expression profiles for genes differentially expressed between S and C lines in (A) Abdomen and (B) Head tissue. Clusters correspond to heat maps displayed on figure 9.

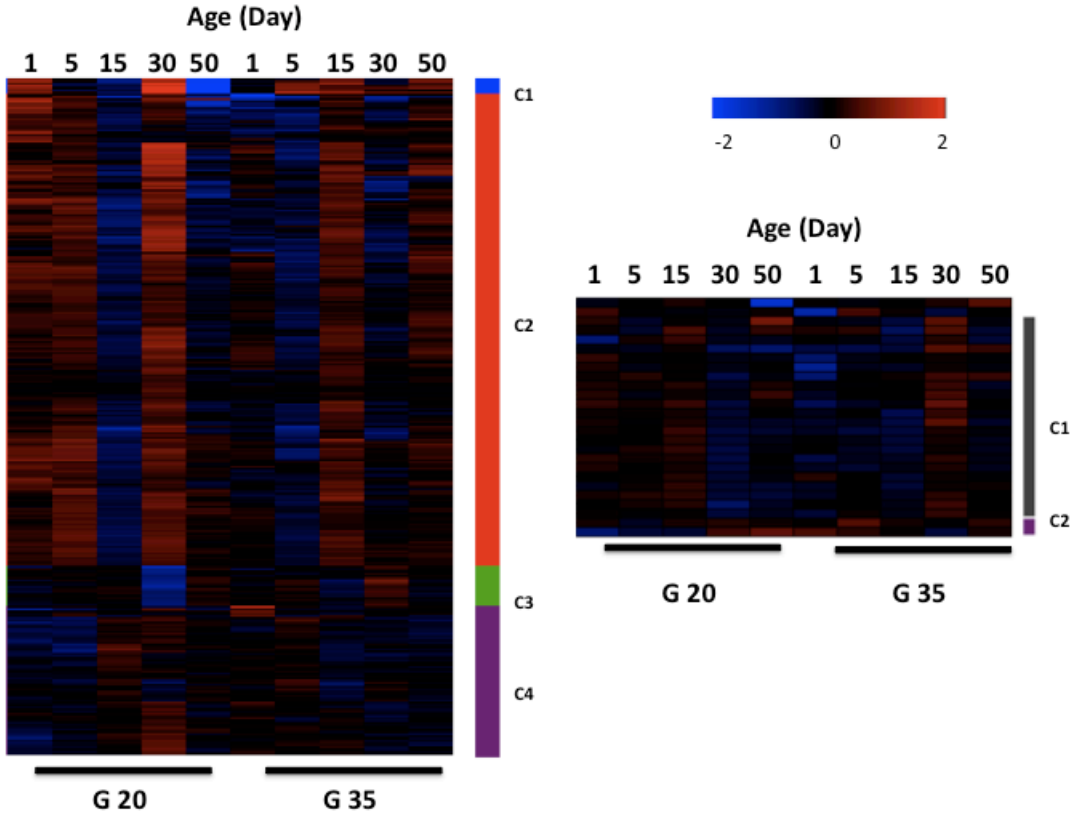


(A) Abdomen



(B) Head

Appendix A. Heat map representation of differentially expressed genes between S and C lines across age and generation for (A) Abdomen and (B) Head tissue. Each column represents an age-class and each row represents the expression pattern of a gene across all age-classes. The ratios of transcript levels between selection and control lines are color coded in red and blue. Red represents an increase in transcript level and blue represents a decrease. Genes in each tissue/effect combination are grouped in two to four clusters (C1-C4) obtained by hierarchical clustering analysis based on similarities in expression profiles.



(A) Abdomen (B) Head

Appendix B. Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 1	Mur2B	X	2B2-2B4	chitin binding
Cluster 1	CG14834	3L	65F5-65F5	None available
Cluster 1	yellow-g2	3L	62D5-62D5	None available
Cluster 1	CG32774	X	4B4-4B4	None available
Cluster 1	CG32972	Unknown	Unknown	None available
Cluster 1	CG4009	3R	89E10-89E10	peroxidase activity
Cluster 1	Defective Chorion 1	X	7C1-7C1	structural constituent of chorion
Cluster 2	CG3209	2R	60B8-60B8	1-acylglycerol-3-phosphate O-acyltransferase activity
Cluster 2	CG10168	3R	95A1-95A1	2-hydroxyacylsphingosine 1-beta-galactosyltransferase activity
Cluster 2	CG10170	3R	95A1-95A1	2-hydroxyacylsphingosine 1-beta-galactosyltransferase activity
Cluster 2	veil	2R	54B16-54B17	5'-nucleotidase activity
Cluster 2	Tm2	3R	88E13-88E13	actin binding
Cluster 2	Tm1	3R	88E12-88E13	actin binding
Cluster 2	wupA	X	16F7-16F7	actin binding
Cluster 2	Adgf-D	3R	87F11-87F11	adenosine deaminase activity
Cluster 2	Adgf-A	3L	75A1-75A1	adenosine deaminase activity; growth factor activity
Cluster 2	CG2767	3R	84E8-84E8	alcohol dehydrogenase (NADP+) activity
Cluster 2	CG8785	2R	49B10-49B11	amino acid transmembrane transporter activity
Cluster 2	CG7888	3L	68A3-68A3	amino acid transmembrane transporter activity
Cluster 2	CG17110	3R	94D13-94D13	aminoacylase activity
Cluster 2	CG8774	3R	87E4-87E5	aminopeptidase activity
Cluster 2	CG31343	3R	93F8-93F8	aminopeptidase activity
Cluster 2	CG31233	3R	93F8-93F8	aminopeptidase activity
Cluster 2	pcl	X	1B2-1B2	aspartic-type endopeptidase activity
Cluster 2	CG4562	3R	92B4-92B4	ATPase activity
Cluster 2	CG33970	3R	97A8-97A10	ATPase activity
Cluster 2	Mlc2	3R	99E1-99E1	ATPase activity
Cluster 2	CG14709	3R	86E11-86E11	ATPase activity
Cluster 2	CG11147	2L	25F4-25F4	ATPase activity, coupled to transmembrane movement of substances
Cluster 2	CG13124	2L	30E1-30E1	binding
Cluster 2	Eip63F-1	3L	63F6-64A1	calcium ion binding
Cluster 2	CG7526	3L	66A5-66A5	calcium ion binding
Cluster 2	Mp20	2R	49F13-49F13	calcium ion binding
Cluster 2	TpnC73F	3L	73E5-73E5	calcium ion binding
Cluster 2	CG4301	X	14C4-14C4	calcium-transporting ATPase activity

Appendix B (Cont.) Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	Pka-C3	3L	72B1-72B2	cAMP-dependent protein kinase activity
Cluster 2	CG3841	2L	30B12-30C1	carboxylesterase activity
Cluster 2	Est-6	3L	69A1-69A1	carboxylesterase activity
Cluster 2	alpha-Est5	3R	84D4-84D5	carboxylesterase activity
Cluster 2	alpha-Est8	3R	84D9-84D9	carboxylesterase activity
Cluster 2	CG15879	3L	62D1-62D1	catalytic activity
Cluster 2	Gasp	3R	83D4-83D4	chitin binding
Cluster 2	ClC-a	3R	86F8-86F8	chloride channel activity
Cluster 2	CG5946	3L	68E1-68E1	cytochrome-b5 reductase activity
Cluster 2	CG8790	3R	87E8-87E8	dicarboxylic acid transmembrane transporter activity
Cluster 2	ome-RA	3L	70E7-70F4	dipeptidyl-peptidase activity
Cluster 2	Zfrp8	2R	60B11-60B12	DNA binding
Cluster 2	Cyp4e1	2R	44D1-44D1	electron carrier activity
Cluster 2	Cyp12a4	3R	91F3-91F3	electron carrier activity
Cluster 2	Cyp6a8	2R	51D1-51D1	electron carrier activity
Cluster 2	Cyp313a3	3R	87B3-87B3	electron carrier activity; oxidoreductase activity
Cluster 2	Cyp4d2	X	2E1-2E1	electron carrier activity; oxidoreductase activity
Cluster 2	Cyp4d14	X	2E1-2E1	electron carrier activity; oxidoreductase activity
Cluster 2	Ir7c	X	7C1-7C1	extracellular-glutamate-gated ion channel activity
Cluster 2	CG7589	3L	74C3-74C3	extracellular-glycine-gated ion channel activity
Cluster 2	CG7910	3R	84E10-84E10	fatty acid amide hydrolase activity
Cluster 2	Fmo-2	2R	42B2-42B2	flavin-containing monooxygenase activity
Cluster 2	mthl3	2R	54B16-54B16	G-protein coupled receptor activity
Cluster 2	Ugt36Ba	2L	36B1-36B1	glucuronosyltransferase activity
Cluster 2	CG11289	2L	27D7-27E1	glucuronosyltransferase activity
Cluster 2	Ugt36Bc	2L	36B1-36B1	glucuronosyltransferase activity
Cluster 2	CG4302	2R	57D1-57D2	glucuronosyltransferase activity
Cluster 2	Ugt58Fa	2R	58F3-58F3	glucuronosyltransferase activity
Cluster 2	Ugt35b	3R	86D5-86D5	glucuronosyltransferase activity
Cluster 2	CG5999	3R	87C8-87C8	glucuronosyltransferase activity
Cluster 2	Ugt86Dg	3R	86D5-86D5	glucuronosyltransferase activity
Cluster 2	Gs2	X	10B11-10B11	glutamate-ammonia ligase activity
Cluster 2	Gyk	3L	61B2-61B2	glycerol kinase activity
Cluster 2	Gpo-1	2R	52C8-52C8	glycerol-3-phosphate dehydrogenase activity
Cluster 2	Glycogenin	2R	57D1-57D1	glycogenin glucosyltransferase activity
Cluster 2	Rab5	2L	22E1-22E1	GTPase activity

Appendix B (Cont.) Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	CG6125	3R	88F4-88F4	high affinity sulfate transmembrane transporter activity
Cluster 2	CG9702	3R	99F9-99F9	high affinity sulfate transmembrane transporter activity
Cluster 2	CG5315	3R	94B3-94B3	hormone binding
Cluster 2	Vha100-5	2L	33A1-33A1	hydrogen-exporting ATPase activity
Cluster 2	Vha100-4	3R	91A5-91A5	hydrogen-exporting ATPase activity, phosphorylative mechanism
Cluster 2	CG2794	2L	21E2-21E2	hydrolase activity
Cluster 2	CG30104	2R	54B17-54B17	hydrolase activity
Cluster 2	Mipp1	3L	73A7-73A9	inositol or phosphatidylinositol phosphatase activity
Cluster 2	CG17027	3L	72C1-72C1	inositol-1(or 4)-monophosphatase activity
Cluster 2	Irk2	3R	95A1-95A1	inward rectifier potassium channel activity
Cluster 2	KaiRIA	3R	92F4-92F4	ionotropic glutamate receptor activity
Cluster 2	Jheh2	2R	55F8-55F8	juvenile hormone epoxide hydrolase activity
Cluster 2	GluRIIE	3R	92F4-92F4	kainate selective glutamate receptor activity
Cluster 2	CG17119	3R	94D10-94D10	L-cystine transmembrane transporter activity
Cluster 2	CG8562	3L	65F11-65F11	metallocarboxypeptidase activity
Cluster 2	CG14820	3L	65D3-65D3	metallocarboxypeptidase activity
Cluster 2	CG18585	2L	28C1-28C1	metallocarboxypeptidase activity
Cluster 2	CG1750	3R	100B8-100B8	methionyl-tRNA formyltransferase activity
Cluster 2	Mlc1	3R	98A14-98A15	microfilament motor activity
Cluster 2	CLIP-190	2L	36C7-36C7	microtubule binding
Cluster 2	CG31100	3R	85C1-85C1	monosaccharide transmembrane transporter activity
Cluster 2	Mbs	3L	72D1-72D1	myosin phosphatase activity
Cluster 2	CG11149	2L	25F3-25F3	N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase activity
Cluster 2	Ast-C	2L	32D2-32D2	neuropeptide hormone activity
Cluster 2	CG1629	4	102B1-102B1	None available
Cluster 2	CG1674	4	102B1-102B1	None available
Cluster 2	CG11076	4	102F6-102F6	None available
Cluster 2	CG1674	4	102B1-102B1	None available
Cluster 2	CG31607	2L	28E7-28E7	None available
Cluster 2	CG31900	2L	28E8-28E9	None available
Cluster 2	CG9267	2L	34C1-34C1	None available
Cluster 2	CG5758	2L	36E5-36E5	None available
Cluster 2	CG3625	2L	21B7-21B7	None available
Cluster 2	CG16820	2L	34B1-34B1	None available
Cluster 2	CG17549	2L	37D4-37D4	None available
Cluster 2	CG18302	2L	31F5-31F5	None available

Appendix B (Cont.) Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	CG7778	2L	29B1-29B1	None available
Cluster 2	RIC-3	2R	57B9-57B12	None available
Cluster 2	CG10307	2R	57F8-57F8	None available
Cluster 2	CG3907	2R	60B3-60B4	None available
Cluster 2	CG7461-RA	2R	56C9-56C10	None available
Cluster 2	swi2	2R	54D5-54D6	None available
Cluster 2	JhI-26	2R	53B1-53B1	None available
Cluster 2	CG33012	2R	49A1-49A1	None available
Cluster 2	CG8008	2R	45B1-45B1	None available
Cluster 2	CG6845	3L	61B1-61B1	None available
Cluster 2	Fie	3L	63F5-63F5	None available
Cluster 2	CG6409	3L	67F1-67F1	None available
Cluster 2	CG7194	3L	66C5-66C5	None available
Cluster 2	CG5059	3L	77C6-77C6	None available
Cluster 2	Zasp66	3L	66D9-66D9	None available
Cluster 2	pst	3L	65F6-65F7	None available
Cluster 2	CG13024	3L	73D1-73D1	None available
Cluster 2	CG31259	3R	85A2-85A2	None available
Cluster 2	fau	3R	86C6-86C6	None available
Cluster 2	Mf	3R	88E2-88E2	None available
Cluster 2	CG8907	3R	89E8-89E8	None available
Cluster 2	CG6921	3R	94B4-94B4	None available
Cluster 2	Tm1	3R	88E12-88E13	None available
Cluster 2	CG18747	3R	84E1-84E1	None available
Cluster 2	CG9400	X	12E7-12E8	None available
Cluster 2	CG12057	X	8C17-8C17	None available
Cluster 2	CG15209	X	9F5-9F5	None available
Cluster 2	CG14439	X	6C11-6C12	None available
Cluster 2	Transporter	Unknown	Unknown	None available
Cluster 2	Probable tRNA(His) guanylyltransferase	Unknown	Unknown	None available
Cluster 2	CG17571	Unknown	Unknown	None available
Cluster 2	FBtr0089969	Unknown	Unknown	None available
Cluster 2	CG30190; CG30193	Unknown	Unknown	None available
Cluster 2	Oatp58Da	2R	58D2-58D2	organic anion transmembrane transporter activity
Cluster 2	vanin-like	X	5E1-5E1	pantetheine hydrolase activity
Cluster 2	GNBP2	3L	75D6-75D6	Gram-negative bacterial cell surface binding

Appendix B (Cont.) Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	CG13160	2R	49A1-49A1	peptidase activity
Cluster 2	CG10051	2R	56D2-56D2	peptidase activity
Cluster 2	CG10062	2R	56D2-56D2	peptidase activity
Cluster 2	CG30043	2R	49A1-49A1	peptidase activity
Cluster 2	CG10211	2L	36F6-36F6	peroxidase activity
Cluster 2	Eb1-RC	2R	42C8-42C8	protein binding
Cluster 2	Pif1B	3R	85B1-85B1	protein binding
Cluster 2	Zasp52	2R	52C4-52C7	protein binding
Cluster 2	tim	2L	23F6-23F6	protein heterodimerization activity
Cluster 2	CkIIbeta	X	10E3-10E3	protein kinase activity
Cluster 2	CG2930	X	3F4-3F4	proton-dependent oligopeptide secondary active transmembrane transporter activity
Cluster 2	CG1889	X	9A3-9A3	receptor binding
Cluster 2	CG6574	3R	86C7-86C7	reduced folate carrier activity
Cluster 2	CG5973	2L	27F4-27F4	retinal binding
Cluster 2	CG10026	2L	37E1-37E1	retinal binding
Cluster 2	CG15611	2R	53F10-53F11	Rho guanyl-nucleotide exchange factor activity
Cluster 2	crc	2L	39C2-39C3	sequence-specific DNA binding transcription factor activity
Cluster 2	CG17475	3R	89F1-89F1	serine-type endopeptidase activity
Cluster 2	CG11911	2L	21B8-21B8	serine-type endopeptidase activity
Cluster 2	CG9372	3L	76B9-76B9	serine-type peptidase activity
Cluster 2	nrv2	2L	27B1-27B2	sodium:potassium-exchanging ATPase activity
Cluster 2	CG15534	3R	99F4-99F4	sphingomyelin phosphodiesterase activity
Cluster 2	CG15531	3R	99E2-99E2	stearoyl-CoA 9-desaturase activity
Cluster 2	Fad2	3L	68A1-68A1	stearoyl-CoA 9-desaturase activity
Cluster 2	CG13284	2L	36B2-36B2	steroid dehydrogenase activity
Cluster 2	Ssl2	3R	98F5-98F5	strictosidine synthase activity
Cluster 2	Cpr62Bb	3L	62B6-62B6	structural constituent of chitin-based cuticle
Cluster 2	Defective Chorion 1	X	7C1-7C1	structural constituent of chorion
Cluster 2	zormin	3L	62C4-62D1	structural constituent of cytoskeleton
Cluster 2	Act87E	3R	87E11-87E11	structural constituent of cytoskeleton
Cluster 2	Scgalpha	2L	29A3-29A3	structural constituent of muscle
Cluster 2	mRpL45	3R	94B6-94B6	structural constituent of ribosome
Cluster 2	Gr59f	2R	59E3-59E3	taste receptor activity
Cluster 2	Vdup1	3L	61B2-61B2	transcription repressor activity
Cluster 2	CG31974	2L	21B3-21B3	transferase activity
Cluster 2	CG33301	2L	31A1-31A1	transferase activity

Appendix B (Cont.) Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 2	CG6908	3R	86E16-86E16	transferase activity
Cluster 2	CG31288	3R	96D1-96D1	transferase activity
Cluster 2	CG31097	3R	96D1-96D1	transferase activity
Cluster 2	CG3106	X	8F6-8F6	transferase activity
Cluster 2	CG32645	X	11E1-11E1	transferase activity
Cluster 2	CG13360	X	1C4-1C4	transferase activity
Cluster 2	CG14160	3L	67D8-67D8	transmembrane transporter activity
Cluster 2	CG6901	3R	89B12-89B12	transmembrane transporter activity
Cluster 2	CG31636	2L	26F5-26F5	transporter activity
Cluster 2	CG32669	X	9F5-9F5	transporter activity
Cluster 2	CG5177	2L	27F3-27F3	trehalose-phosphatase activity
Cluster 2	up	X	12A7-12A7	tropomyosin binding
Cluster 2	CG1550	2R	43E18-43E18	tubulin-tyrosine ligase activity
Cluster 2	CAP	2R	46F9-47A1	vinculin binding
Cluster 2	CG3091	X	2F2-2F2	vitamin E binding; transporter activity
Cluster 2	Drip	2R	47F11-47F11	water transmembrane transporter activity
Cluster 2	Ance	2L	34E2-34E2	zinc ion binding
Cluster 3	Msp-300	2L	25C6-25C10	actin binding
Cluster 3	Syt1	2L	23A6-23B1	calcium ion binding
Cluster 3	Ets96B	3R	96A22-96A22	DNA binding
Cluster 3	CG11131	3L	80B2-80B2	None available
Cluster 3	CG32815	X	1D2-1D2	None available
Cluster 3	PIP82	X	7F3-7F3	None available
Cluster 3	CG9782	X	14F2-14F2	None available
Cluster 3	CG15034	X	7B2-7B2	None available
Cluster 3	CG11584	X	12E2-12E2	None available
Cluster 3	CG11473-RA	Unknown	Unknown	None available
Cluster 3	Fps85D	3R	85D13-85D15	protein tyrosine kinase activity
Cluster 3	Crg-1	X	3F1-3F2	RNA polymerase II transcription factor activity
Cluster 3	ind	3L	71B2-71B2	sequence-specific DNA binding transcription factor activity
Cluster 3	Cpr67Fa1	3L	67F1-67F1	structural constituent of chitin-based cuticle
Cluster 3	Cpr31A	2L	31A1-31A1	structural constituent of chitin-based cuticle
Cluster 3	CG15439	2L	24F3-24F3	zinc ion binding
Cluster 3	erm	2L	22B6-22B7	zinc ion binding
Cluster 3	CG7271	3L	75D2-75D2	zinc ion binding

Appendix B (Cont.) Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 4	kel	2L	36E5-36E6	actin binding
Cluster 4	bif	X	10D4-10D5	actin binding
Cluster 4	Ama	3R	84A5-84A5	antigen binding
Cluster 4	CG31961	2L	24D8-24D8	binding
Cluster 4	CG15443	2L	24F3-24F3	binding
Cluster 4	CG5756	2R	55B5-55B5	chitin binding
Cluster 4	CG5611	3R	98A12-98A13	delta5-delta2,4-dienoyl-CoA isomerase activity
Cluster 4	dnk	3R	91E2-91E2	deoxynucleoside kinase activity
Cluster 4	Ada1-1	2L	33B5-33B5	DNA binding
Cluster 4	CG4570	3R	86C7-86C7	DNA binding
Cluster 4	Sry-beta	3R	99D3-99D3	DNA binding
Cluster 4	Ada1-2	2L	33B5-33B5	DNA binding
Cluster 4	CG14232	X	18E1-18E1	fatty-acyl-CoA binding
Cluster 4	CG32412	3L	64F4-64F5	glutaminyl-peptide cyclotransferase activity
Cluster 4	CG1271	3L	63A5-63A5	glycerol kinase activity
Cluster 4	Jheh2	2R	55F8-55F8	juvenile hormone epoxide hydrolase activity
Cluster 4	CG14656	3R	82D5-82D6	ligand-dependent nuclear receptor binding
Cluster 4	Las	3L	77C2-77C3	lipoic acid synthase activity
Cluster 4	CG33156	2R	50B1-50B1	NAD+ kinase activity
Cluster 4	CG4785	2L	21F1-21F1	None available
Cluster 4	CG3862	2L	21E2-21E2	None available
Cluster 4	CG13982	2L	26D1-26D1	None available
Cluster 4	CG5327	2R	55E2-55E2	None available
Cluster 4	CG7686	2R	47C3-47C3	None available
Cluster 4	CG8179; CG34318	2R	52A3-52A4	None available
Cluster 4	CG34219	2R	44F7-44F7	None available
Cluster 4	CG9186	3L	61F6-61F6	None available
Cluster 4	Rcd5	3L	64A4-64A4	None available
Cluster 4	CG5274	3L	77C2-77C2	None available
Cluster 4	CG7852	3L	62A3-62A3	None available
Cluster 4	CG9356	3R	85D15-85D15	None available
Cluster 4	beat-IIa	3R	90A2-90A3	None available
Cluster 4	CG7175	3R	90F7-90F8	None available
Cluster 4	CG8031	3R	87D8-87D8	None available
Cluster 4	CG17003	X	19A4-19A4	None available
Cluster 4	CG32779	X	3F2-3F2	None available

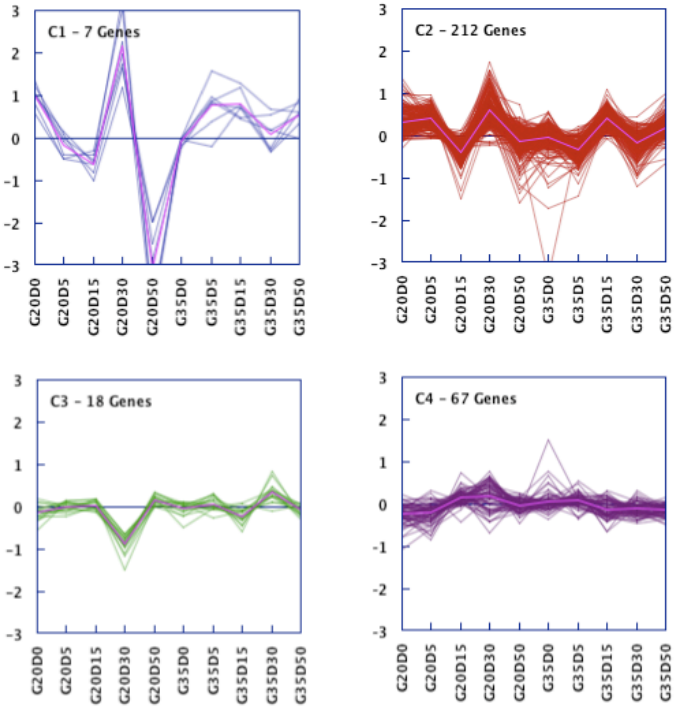
Appendix B (Cont.) Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Abdomen tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 4	CG4068	X	4D6-4D7	None available
Cluster 4	CG9053	X	13A8-13A8	None available
Cluster 4	CG6506	X	16E1-16E1	None available
Cluster 4	CG13373	X	1B4-1B4	None available
Cluster 4	CG14220	X	18D3-18D3	None available
Cluster 4	CG7206	X	16F6-16F6	None available
Cluster 4	CG33224	X	8C4-8C4	None available
Cluster 4	CG41457-RA	Unknown	Unknown	None available
Cluster 4	Semaphorin-1A	Unknown	Unknown	None available
Cluster 4	dsh	X	10B4-10B5	Notch binding
Cluster 4	CG7914	X	18A7-18A7	oxidoreductase activity
Cluster 4	CG9531	2L	26D7-26D7	oxygen-dependent protoporphyrinogen oxidase activity
Cluster 4	CG1236	3R	83C1-83C1	phosphoglycerate dehydrogenase activity
Cluster 4	CG30184	2R	59F1-59F1	phosphotransferase activity
Cluster 4	ial	2L	32B2-32B2	protein serine/threonine kinase activity
Cluster 4	CG17746	3L	63C1-63C1	protein serine/threonine phosphatase activity
Cluster 4	CG7024	X	4C14-4C14	pyruvate dehydrogenase (acetyl-transferring) activity
Cluster 4	Snr1	3R	83A4-83A4	RNA polymerase II transcription factor activity
Cluster 4	D	3L	70D3-70D3	sequence-specific DNA binding transcription factor activity
Cluster 4	Doc1	3L	66F2-66F3	sequence-specific DNA binding transcription factor activity
Cluster 4	CG30286	2R	57E9-57E10	serine-type endopeptidase activity
Cluster 4	CG31200	3R	92F10-92F10	serine-type endopeptidase activity
Cluster 4	CG1295	3L	64A10-64A10	sphingomyelin phosphodiesterase activator activity
Cluster 4	mam	2R	50C23-50D3	transcription coactivator activity
Cluster 4	CG5366	2L	31D10-31D10	transcription factor binding
Cluster 4	jing	2R	42C1-42B2	transcription repressor activity
Cluster 4	CG12714	X	11D11-11E1	transferase activity
Cluster 4	Uch	2L	22D4-22D4	ubiquitin thiolesterase activity
Cluster 4	CG15439	2L	24F3-24F3	zinc ion binding
Cluster 4	CG8003	3L	67E6-67E6	zinc ion binding
Cluster 4	CG31457	3R	94E1-94E1	zinc ion binding

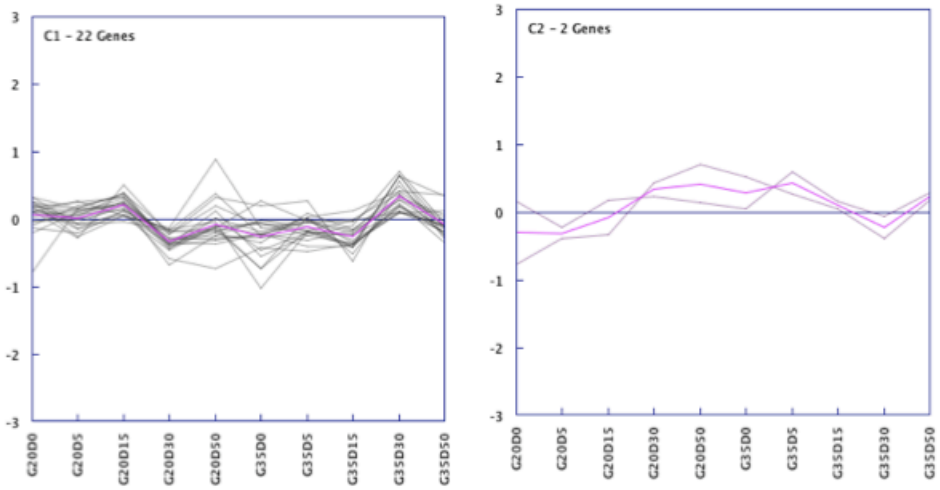
Appendix C. Genes with significantly different expression between S and C lines across age and generation (line*age*gen) interaction in Head tissue.

Cluster	Gene Name/Symbol	Location		Molecular Function
Cluster 1	betaggt-II	2L	23C1-23C1	CAAX-protein geranylgeranyltransferase activity
Cluster 1	CG17712	2L	22B2-22B2	oxidoreductase activity
Cluster 1	CG9150	2L	26B4-26B4	oxidoreductase activity
Cluster 1	Tg	2L	28D3-28D3	protein-glutamine gamma-glutamyltransferase activity
Cluster 1	Spn28D	2L	28D3-28D3	serine-type endopeptidase inhibitor activity
Cluster 1	CG13090	2L	29D4-29D4	Mo-molybdopterin cofactor sulfurase activity
Cluster 1	bl	2R	57A6-57A7	mRNA binding
Cluster 1	CG15611	2R	53F10-53F11	Rho guanyl-nucleotide exchange factor activity
Cluster 1	CG9149	3L	61F5-61F5	acetyl-CoA C-acetyltransferase activity
Cluster 1	CG1275	3L	62D4-62D4	electron carrier activity
Cluster 1	Faa	3L	64A5-64A5	fumarylacetoacetase activity
Cluster 1	CG4484	3L	67A3-67A3	sucrose:hydrogen symporter activity
Cluster 1	CG11796	3L	77C3-77C3	4-hydroxyphenylpyruvate dioxygenase activity
Cluster 1	CG1213	3R	83C5-83C5	glucose transmembrane transporter activity
Cluster 1	CG1115	3R	82F6-82F6	None available
Cluster 1	CG1092	3R	82A1-82A1	None available
Cluster 1	CG3940	3R	85F12-85F12	carbonate dehydratase activity
Cluster 1	Spn5	3R	88E3-88E3	serine-type endopeptidase inhibitor activity
Cluster 1	CG2003	3R	100E1-100E1	high affinity inorganic phosphate:sodium symporter activity
Cluster 1	Dsor1	X	8D2-8D3	MAP kinase kinase activity
Cluster 1	CG14215	X	18D13-18D13	None available
Cluster 2	CG17374	3L	Unknown	fatty acid synthase activity
Cluster 2	net	2L	21B1-21B1	RNA polymerase II transcription factor activity
No cluster	Hsp68	3R	95D11-95D11	unfolded protein binding
No cluster	CG14545	3R	96F3-96F3	None available

Appendix D. Gene expression profiles for genes differentially expressed between S and C lines with age and generation (line*age*gen) in (A) Abdomen and (B) Head tissue. Clusters correspond to heat maps displayed on Appendix A.



(A) Abdomen



(B) Head